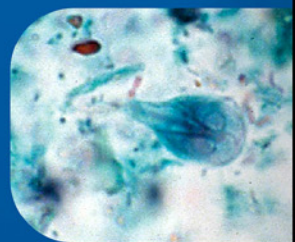
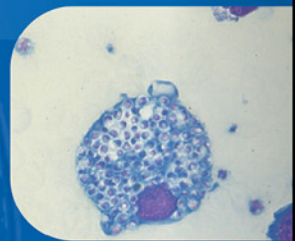
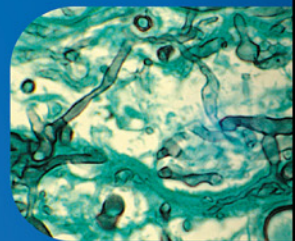


SEVENTH EDITION

TEXTBOOK OF

Diagnostic Microbiology

Connie R. Mahon
Donald C. Lehman



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Textbook of Diagnostic Microbiology

SEVENTH EDITION

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Preface

While we were preparing the seventh edition of the *Textbook of Diagnostic Microbiology*, a never-before-seen virus was reported in China in December 2019 and spread quickly around the world, reaching Europe and the United States in January 2020. This novel virus was identified as the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), the cause of coronavirus disease 2019. This disease emphasizes how quickly infectious agents can spread and the significance of the work of microbiologists and other health care professionals. Newly recognized pathogens continue to emerge while previously known pathogens evolve to plague society.

The field of diagnostic microbiology continues to change dramatically and becomes more complex. Hence, we look ahead to the future needs of the next generation of medical laboratory practitioners. Methods for the detection and identification of infectious agents continue to evolve, and more laboratories are replacing traditional methods (e.g., biochemical identification of bacteria and cell cultures for viruses) with molecular biology assays. Therefore we continue to update and expand this topic in Chapter 11, where we discuss matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry and nucleic acid–based identification methods that include probes, amplification assays, and gene sequencing. This also includes nanomedicine in diagnosing infectious diseases. Bacteria continue to become more drug resistant, so science must keep pace by rapidly determining a bacterium’s susceptibility pattern and finding better ways to treat life-threatening infections. In Chapter 12, we discuss common means by which bacteria develop resistance to agents and present the most used and cutting-edge antimicrobial agents. We follow this in Chapter 13 by examining the best methods to assay for drug sensitivity and resistance.

With the advent of these emerging molecular technology innovations, scientific discoveries, and gene-based techniques, medical microbiology education in medical laboratory science programs has also undergone dramatic changes. In these highly specialized and rapidly advancing areas of science and biotechnology, we have taken the lead to constantly look for innovative ways to effectively deliver the textbook contents in ways that they are current, appropriate, absorbable, and applicable.

As in previous editions, the seventh edition maintains the characteristic features of a well-designed and organized textbook. We maintain the building-block approach to learning, critical thinking, and problem solving, attributes that students of medical laboratory science and medical laboratory technology, entry-level clinical laboratory scientists, and others have found valuable and effective. The primary goal of the *Textbook of Diagnostic Microbiology* is to provide a strong foundation for medical laboratory science students, entry-level practitioners, and other health care professionals; therefore discussions on organisms are limited to those that are medically

important and commonly encountered, as well as new and re-emerging pathogens. A feature that sets our textbook apart is the large number of quality full-color photographs and photomicrographs. The text also provides students and other readers with valuable learning tools, such as summary tables, flowcharts, and descriptive illustrations, to help them comprehend the vast amount of information and reinforce learning. In response to our readers’ needs, we continued our efforts to enhance these features that have made this textbook user-friendly. All our contributing authors are experts in the field of microbiology, either practicing laboratory scientists, infectious disease specialists, researchers, or educators. Their expertise helps us to have current and accurate information.

Organization

Part I remains the backbone of the textbook, providing important background information; Part II focuses on laboratory identification of etiologic agents organized taxonomically; and Part III on the *organ system approach*—the clinical and laboratory diagnoses of infectious diseases at various body sites.

Part 1 presents basic principles and concepts of diagnostic microbiology, including quality assurance, providing students with a firm theoretic foundation. Chapters 7 (Microscopic Examination of Materials From Infected Sites) and 8 (Use of Colony Morphology for the Presumptive Identification of Microorganisms) still play a vital role in this text. These two chapters help students and practitioners who may have difficulty recognizing bacterial morphology on direct smear preparations and colony morphology on primary culture plates develop these skills with the use of color photomicrographs of stained direct smears and cultures from clinical samples. These two chapters also illustrate how microscopic and colony morphology of organisms can aid in the initial identification of the bacterial isolate. Chapter 9 introduces the student/reader to the principles behind various biochemical methods for identification of gram-negative bacteria. This chapter contains several color photographs to help our readers understand the principles and visualize interpretations of these important tests.

Part 2 highlights methods for the identification of clinically significant isolates that include bacteria, fungi, parasites, and viruses. Although diseases caused by the infectious agents are discussed, the emphasis is on the characteristics and methods used to isolate and identify each group of pathogens. Numerous tables summarize the major features of organisms and use schematic diagrams to show the relationships and differences among similar or closely related species. Chapters devoted to anaerobic bacterial species, medically important fungi, parasites, and viruses affirm the

significance of these agents. Chapter 29 includes a discussion on viral pathogens, including newly discovered Zika virus, SARS-CoV-2, and avian influenza virus. Chapter 31 describes biofilm—an increasingly complex entity. It has become evident that microbial biofilms are involved in the pathogenesis of several human diseases and may be a contributing factor of antimicrobial therapy failure.

The organ system approach in Part 3 has been the foundation of the *Textbook of Diagnostic Microbiology* and provides an opportunity for students and other readers to “pull things together.” The chapters in Part 3 begin with the anatomic considerations of the organ system to be discussed and the role of the usual microbiota found at a particular site in the pathogenesis of a disease. It is important for students to be knowledgeable about the usual inhabitants at a body site before they can appreciate the significance of the infectious agents they are most likely to encounter. These chapters also discuss proper specimen collection and processing.

Pedagogic features

As in previous editions, the “Case in Point” case study found in all chapters introduces the reader to an important pathogen, infectious disease, concept, or principle that is discussed in the chapter text and is used to lead the learner to the main context discussed in the chapter. The Case in Point is followed by “Issues to Consider.” These points are presented in a bulleted format, and learners are asked to think about them as they read the chapter. We use case studies to enhance problem-solving and critical-thinking skills. The case studies describe clinical and laboratory findings, providing students with opportunities to correlate these observations with possible etiologic agents. In most cases, the cause of the illness is not disclosed in the case study; rather, it is presented elsewhere in the chapter to give students the opportunity to independently determine the explanations. In Part 3, the case studies also help students apply the knowledge they acquired from Parts 1 and 2.

“Case Check” boxes aim to reinforce understanding of the content or concept within the context of the Case in Point at the beginning of the chapter or case study at the beginning of a section within the chapter. The Case Check highlights a

specific point in the text and intends to help the learner connect the dots between the points under discussion, as illustrated by the case study.

To further reinforce learning, identification tables, flowcharts, and featured illustrations have been updated, and new ones have been added. Learning objectives and a list of key terms are also provided at the beginning of each chapter. The list of key terms includes abbreviations used in the text so that students can easily find them. At the end of each chapter, readers will find “Points to Remember” and “Learning Assessment Questions,” which help reinforce comprehension and understanding of important concepts. Points to Remember includes a bulleted list of important concepts and highlights that the reader should have learned from the chapter. We also include answers to all the learning assessment questions found in each chapter.

Ancillaries for instructors and students

As in the case of previous editions, we continue to offer a variety of instructor ancillaries specifically geared for this book. For instructors, the Evolve website includes a test bank containing more than 1200 questions. It also includes an electronic image collection and PowerPoint slides. For students, the Evolve website will include a laboratory manual like it always has, which contains new case studies and student review questions. It also includes appendices, which provide information on laboratory media commonly used for the cultivation of bacteria and fungi as well as detailed protocols for selected clinical microbiology laboratory tests.

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We are grateful to all contributing authors, students, instructors, and many other individuals, who have all made invaluable suggestions and comments on ways to improve this edition.

Connie R. Mahon
Donald C. Lehman

Contents

Part 1: Introduction to clinical microbiology

1. Bacterial cell structure, physiology, metabolism, and genetics 2
Connie R. Mahon
2. Host-parasite interaction 25
Donald C. Lehman
3. The laboratory role in infection control 50
Marie Ciacco Tsivitis and Connie R. Mahon
4. Control of microorganisms: disinfection, sterilization, and microbiology safety 65
Michelle M. Jackson
5. Performance improvement in the microbiology laboratory 102
Connie R. Mahon and Olga Kochar
6. Specimen collection and processing 123
Lauren Roberts
7. Microscopic examination of materials from infected sites 139
Connie R. Mahon
8. Use of colony morphology for the presumptive identification of microorganisms 169
Connie R. Mahon
9. Biochemical identification of gram-negative bacteria 183
Donald C. Lehman
10. Immunodiagnosis of infectious diseases 199
Donald C. Lehman
11. Applications of molecular diagnostics 227
Steven D. Mahlen, Vijay Parashar, and Donald C. Lehman
12. Antibacterial mechanisms of action and bacterial resistance mechanisms 250
Brandon C. Ellis and Donald C. Lehman
13. Antimicrobial susceptibility testing 271
Paula C. Mister and Donald C. Lehman

Part 2: Laboratory identification of significant isolates

14. *Staphylococcus* and similar organisms 308
Lindsey E. Nielsen and Jed M. Doxtater
15. *Streptococcus*, *Enterococcus*, and other catalase-negative, gram-positive cocci 324
Kalavati Suvana and Connie R. Mahon

16. Aerobic gram-positive bacilli 347
Steven D. Mahlen and Amanda T. Harrington
17. *Neisseria* species and *Moraxella catarrhalis* 371
Lauren Roberts
18. *Haemophilus*, HACEK group, *Legionella*, and other fastidious gram-negative bacilli 390
Tori Enomoto and A. Christian Whelen
19. Enterobacterales 417
Denene Lofland, Connie R. Mahon, and Donald C. Lehman
20. *Vibrio*, *Aeromonas*, *Campylobacter*, and *Campylobacter*-like species 455
Deborah A. Josko
21. Nonfermenting and miscellaneous gram-negative bacilli 474
Yousif Barzani
22. Anaerobes of clinical importance 496
Nancy Gouin
23. The spirochetes 533
Amy M. Woron and A. Christian Whelen
24. *Chlamydia*, *Rickettsia*, and similar organisms 543
Donald C. Lehman
25. *Mycoplasma* and *Ureaplasma* 558
Donald C. Lehman and Connie R. Mahon
26. *Mycobacterium tuberculosis* and nontuberculous mycobacteria 570
Donald C. Lehman
27. Medically significant fungi 597
Connie F. Cañete-Gibas and Nathan P. Wiederhold
28. Diagnostic parasitology 639
Lauren Roberts and Connie R. Mahon
29. Clinical virology 708
Kevin M. McNabb and Vijay Parashar
30. Agents of bioterror and forensic microbiology 753
Christopher J. Woolverton and Donald C. Lehman
31. Biofilms: architects of disease 776
Donald C. Lehman

Part 3: Laboratory diagnosis of infectious diseases: an organ system approach to diagnostic microbiology

32. Upper and lower respiratory tract infections 791
Susan M. Pacheco and James L. Cook

- 33. Skin and soft tissue infections 832**
Nina M. Clark
- 34. Gastrointestinal infections and food poisoning 869**
Alfredo J. Mena Lora and Connie R. Mahon
- 35. Infections of the central nervous system 889**
Sumathi Nambiar and Kalavati Suvarna
- 36. Bacteremia and sepsis 904**
Paula C. Mister and Donald C. Lehman
- 37. Urinary tract infections 922**
Lindsey E. Nielsen
- 38. Genital infections and sexually transmitted infections 942**
Denene Lofland
- 39. Infections in special populations 974**
Paula C. Mister and Donald C. Lehman
- 40. Zoonotic diseases 984**
David H. Nielsen and Lindsey E. Nielsen
- 41. Ocular infections 1001**
Gail Reid
- Appendix A** Answers to learning assessment questions 1023
- Appendix B** Selected bacteriologic culture media* 1044.e1
- Appendix C** Selected mycology culture media and stains* 1044.e19
- Appendix D** Selected procedures* 1044.e23
- Glossary 1045
- Index 1074

*Available online on the Evolve Resources site (<http://evolve.elsevier.com/Mahon/microbiology/>).

PART

1

Introduction to clinical microbiology

Bacterial cell structure, physiology, metabolism, and genetics

Connie R. Mahon

CHAPTER OUTLINE

Overview of the microbial world, 4

- Bacteria, 4
- Parasites, 4
- Fungi, 4
- Viruses, 6

Classification/Taxonomy, 6

- Nomenclature, 6
- Classification by phenotypic and genotypic characteristics, 7
- Classification by cellular type: prokaryotes, eukaryotes, and archaea, 7

Comparison of prokaryotic and eukaryotic cell structure, 7

- Prokaryotic cell structure, 7
- Eukaryotic cell structure, 10
- Cytoplasmic structures, 7

Bacterial morphology, 11

- Microscopic shapes, 11
- Common stains used for microscopic visualization, 11

Microbial growth and nutrition, 13

- Nutritional requirements for growth, 14
- Environmental factors influencing growth, 15
- Bacterial growth, 15

Bacterial biochemistry and metabolism, 16

- Metabolism, 16
- Fermentation and respiration, 16
- Biochemical pathways from glucose to pyruvic acid, 17
- Anaerobic utilization of pyruvic acid (Fermentation), 17
- Aerobic utilization of pyruvate (Oxidation), 18
- Carbohydrate utilization and lactose fermentation, 19

Microbial genetics, 19

- Anatomy of a DNA and RNA molecule, 19
- Terminology, 21
- Genetic elements and alterations, 21
- Mechanisms of gene transfer, 22

Bibliography, 24

OBJECTIVES

After reading and studying this chapter, you should be able to:

1. Differentiate among archaeal, prokaryotic (bacterial), and eukaryotic cell types.
2. Describe microbial classification (taxonomy).
3. Accurately apply the rules of scientific nomenclature for bacterial names.
4. List and define five methods used by epidemiologists to subdivide bacterial species.
5. Contrast prokaryotic and eukaryotic cytoplasmic and cell wall structures and functions.
6. Compare the cell walls of gram-positive and gram-negative bacteria.
7. Explain why acid-fast bacteria do not stain well with the Gram stain.
8. Justify the use of the following stains in the diagnostic microbiology laboratory: Gram stain, acid-fast stains (Ziehl-Neelsen, Kinyoun, auramine-rhodamine), acridine orange, methylene blue, calcofluor white, lactophenol cotton blue, and India ink.
9. Describe the nutritional requirements for bacterial growth, and define the categories of media used for culturing bacteria in the laboratory.
10. Define the atmospheric requirements of obligate aerobes, microaerophiles, facultative anaerobes, obligate anaerobes, aerotolerant anaerobes, and capnophilic bacteria.
11. Differentiate an aerotolerant anaerobe from facultative and obligate anaerobes.
12. Describe the stages in the growth (i.e., growth curve) of bacterial cells.
13. Explain the importance of understanding microbial metabolism in clinical microbiology.
14. Differentiate between fermentation and oxidation (aerobic respiration).
15. Compare the three biochemical pathways that bacteria use to convert glucose to pyruvate.
16. Differentiate the two types of glucose fermentation that explain positive results with the methyl red and Voges-Proskauer tests.
17. Define the following genetic terms: *genotype*, *phenotype*, *constitutive*, *inducible*, *replication*, *transcription*, *translation*, *genome*, *chromosome*, *plasmids*, *insertion sequence element*, *transposon*, *point mutations*, *frameshift mutations*, *missense mutations*, *nonsense mutations*, and *recombination*.
18. Discuss the development and transfer of antimicrobial resistance in bacteria.

19. Differentiate among the mechanisms of transformation, transduction, and conjugation in the transfer of genetic material from one bacterium to another.
20. Define the terms *bacteriophage*, *lytic phage*, *lysogeny*, and *temperate phage*.
21. Define the term *restriction endonuclease enzyme* and explain how it applies to the clinical microbiology laboratory.

KEY TERMS

Acid fast	Microaerophilic
Aerobic respiration (oxidation)	Minimal medium
Aerotolerant anaerobes	Mycelia
Anticodon	Nomenclature
Archaea	Nutrient media
Autotroph	Obligate aerobes
Bacteria	Obligate anaerobes
Bacteriophage	Pathogenic bacteria
Capnophilic	Phenotype
Capsule	Phyla
Codon	Pili
Competent	Plasmids
Conjugation	Pleomorphic
Differential media	Prokaryote
Dimorphic	Protein expression
Eukarya	Psychrophile
Eukaryote	Replication
Facultative anaerobes	Restriction enzymes
Family	Selective media
Fermentation	Species
Fimbriae	Spore
Flagella	Strains
Fusiform	Taxa
Genotype	Taxonomy
Genus	Temperate
gram-negative	Thermophile
gram-positive	Transcription
Halophile	Transduction
Heterotroph	Transformation
Hyphae	Translation
Krebs cycle	Transport medium
Lysogeny	Virion
Mesophiles	

Case in point

A 4-year-old female patient presents with symptoms of redness, burning, and light sensitivity in both eyes. She also complains of her eyelids sticking together because of exudative discharge. A Gram stain of the conjunctival exudates (product of acute inflammation with white blood cells and fluid) shows gram-positive intracellular and extracellular, faint-staining, coccobacillary bacteria. The organisms appear to have small, clear, nonstaining “halos” surrounding each cell. This clear area is noted to be between the stained organism and the amorphous (no definite form; shapeless) background material. The Gram stain of the quality control organisms *Staphylococcus aureus* (gram-positive) and *Escherichia coli* (gram-negative) reveals gram-positive reactions for both organisms.

Issues to consider

After reading the patient's case history, consider:

- Role of microscopic morphology in presumptive identification
- Significance of observable cellular structures
- Importance of quality control in assessing and interpreting direct smear results
- Unique characteristics of organisms, such as cellular structures, in initiating infection and disease in hosts

Microbial inhabitants have evolved to survive in various ecologic niches (place or location) and habitats (organism's location and where its resources may be found). Some grow rapidly, and others grow slowly. Some can replicate with a minimal number of nutrients present, whereas others require complex, enriched nutrients to survive. Variation exists in atmospheric growth conditions and temperature requirements. This diversity exists in microorganisms that inhabit the human body as normal biota (flora), as opportunistic pathogens, or as true pathogens. Each microbe has its own physiology and metabolic pathways that allow it to survive in its particular habitat. A main role of the clinical microbiologist is to isolate, identify, and analyze the bacteria that cause disease in humans (pathogens). Recent advances in molecular biology methods, such as nucleic acid amplification, nucleic acid sequencing, and matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry, have shifted identification away from traditional biochemical testing for identification of bacterial isolates. Nevertheless, knowledge of microbial structure and physiology is extremely important to clinical microbiologists in the following areas:

- Microscopic characterization of organisms
- Recovery of organisms in culture from patient specimens
- Characterization and identification of organisms after they have been isolated
- Interpretation of antimicrobial susceptibility patterns

The first step in bacterial identification is often microscopic characterization—describing the shape of the organism and its staining characteristics, which are based on the cell wall structure. Understanding the growth requirements of a particular bacterium enables the microbiologist to select the appropriate medium for primary culture and optimize the recovery and isolation of the pathogen. This is followed by observation of the metabolic biochemical differences among organisms that form the basis for many bacterial identification systems in use today. The cell structure and biochemical pathways of an organism also determine its susceptibility to various antimicrobial agents. The ability of microorganisms to change rapidly, acquire new genes, and undergo mutations presents continual challenges to clinical microbiologists as they isolate and characterize microorganisms associated with infectious diseases in humans.

This chapter provides a review of the structure, physiology, metabolism, and genetics of prokaryotic and eukaryotic cells. It also gives examples of common stains used to microscopically visualize microorganisms. Each topic in this chapter emphasizes to clinical microbiologists the inherent importance of their efforts to culture, identify, and characterize the microbes that cause disease in humans.

Overview of the microbial world

The study of microorganisms by the Dutch biologist and lens maker Anton van Leeuwenhoek has evolved immensely from its early historic beginnings. Because of Leeuwenhoek's discovery of what he affectionately called *wee beasties* and *animalcules* in a water droplet with his home-made microscope, the scientific community acknowledged him as the "father of protozoology and bacteriology." Today, we know that there are enormous numbers of microbes in, on, and around us in our environment. The vast majority of these microbes do not cause disease. This textbook focuses on microbes that are associated with human disease.

Bacteria

Bacteria are unicellular organisms that are classified as **prokaryotes** (Greek: before kernel [nucleus]). They lack a nuclear membrane and a true nucleus, mitochondria, an endoplasmic reticulum (ER), and Golgi bodies. The absence of the preceding bacterial cell structures differentiates them from **eukaryotes** (Greek *eu*: well or good; Greek *karyon*: kernel). [Table 1.1](#) compares prokaryotic and eukaryotic cell organization; [Fig. 1.1](#) shows both types of cells.

Parasites

Certain eukaryotic parasites (organisms that live at the expense of their hosts) exist as unicellular organisms of microscopic size, whereas others are multicellular organisms. Protozoa are unicellular organisms within the kingdom Protista that obtain their nutrition through ingestion. Some are capable of locomotion (motile), whereas others are non-motile. They are categorized by their locomotive structures: flagella (Latin: whiplike), pseudopodia (Greek: false feet), or cilia (Latin: eyelash). Many multicellular parasites can be quite large; for example, tapeworms may be 7 to 10m long (see [Chapter 28](#)).

Fungi

Fungi are heterotrophic (cannot produce all of its nutrients) eukaryotes that obtain nutrients through absorption. Most fungi are multicellular, and many can reproduce sexually and asexually. Multicellular fungi are composed of filaments called **hyphae** that interweave to form mats called **mycelia**. Yeasts are unicellular fungi that reproduce asexually. "True" yeasts do not form hyphae or mycelia. Molds are filamentous forms that can reproduce asexually and sexually. Certain fungi can assume both morphologies (yeast and hyphae/mycelial

Table 1.1 Comparison of prokaryotic and eukaryotic cell organization

Characteristic	Prokaryote	Eukaryote
Typical size	0.4–2 μ m in diameter 0.5–5 μ m in length	10–100 μ m in diameter >10 μ m in length
Nucleus	No nuclear membrane; nucleoid region of the cytosol	Membrane-bound nucleus
Genome		
Location	In the nucleoid, at the mesosome	In the nucleus
Chromosomal DNA	Circular; complexed with RNA	Linear; complexed with basic histones and other proteins
Extrachromosomal circular DNA	Plasmids, small circular molecule of DNA in the cytoplasm containing accessory information; most commonly found in gram-negative bacteria; each carries genes for its own replication; can confer resistance to antimicrobial agents	In mitochondria, chloroplasts, and cytoplasm
Reproduction	Asexual (binary fission)	Sexual and asexual
Membrane-bound organelles	Absent	All
Golgi bodies	Absent in all	Present in some
Lysosomes	Absent in all	Present in some; contain hydrolytic enzymes
Endoplasmic reticulum	Absent in all	Present in all; lipid synthesis, transport
Mitochondria	Absent in all	Present in most
Chloroplasts for photosynthesis	Absent in all	Present in algae and plants
Ribosomes: site of protein synthesis (nonmembranous)	Present in all	Present in all
Size	70S consisting of 50S and 30S subunits	80S consisting of 60S and 40S subunits
Electron transport for energy	In the cell membrane; no mitochondria present	In the inner membrane of mitochondria and chloroplasts

Table 1.1 Comparison of prokaryotic and eukaryotic cell organization—cont'd

Characteristic	Prokaryote	Eukaryote
Sterols in cytoplasmic membrane	Absent except in Mycoplasmataceae	Present
Plasma membrane	Phospholipid bilayer; lacks carbohydrates	Phospholipid bilayer; also contains glycolipids and glycoproteins
Cell wall, if present	Peptidoglycan in most bacteria	Cellulose, phenolic polymers, lignin (plants), chitin (fungi), other glycans (algae)
Glycocalyx	Present in many as an organized capsule or unorganized slime layer	Present; some animal cells
Cilia	Absent	Present; see description of flagella
Flagella, if present	Simple flagella; composed of polymers of flagellin; movement by rotary action at the base; spirochetes have MTs	Complex cilia or flagella; composed of MTs and polymers of tubulin with dynein connecting MTs; movement by coordinated sliding MTs
Pili and fimbriae	Present	Absent

MT, Microtubule.

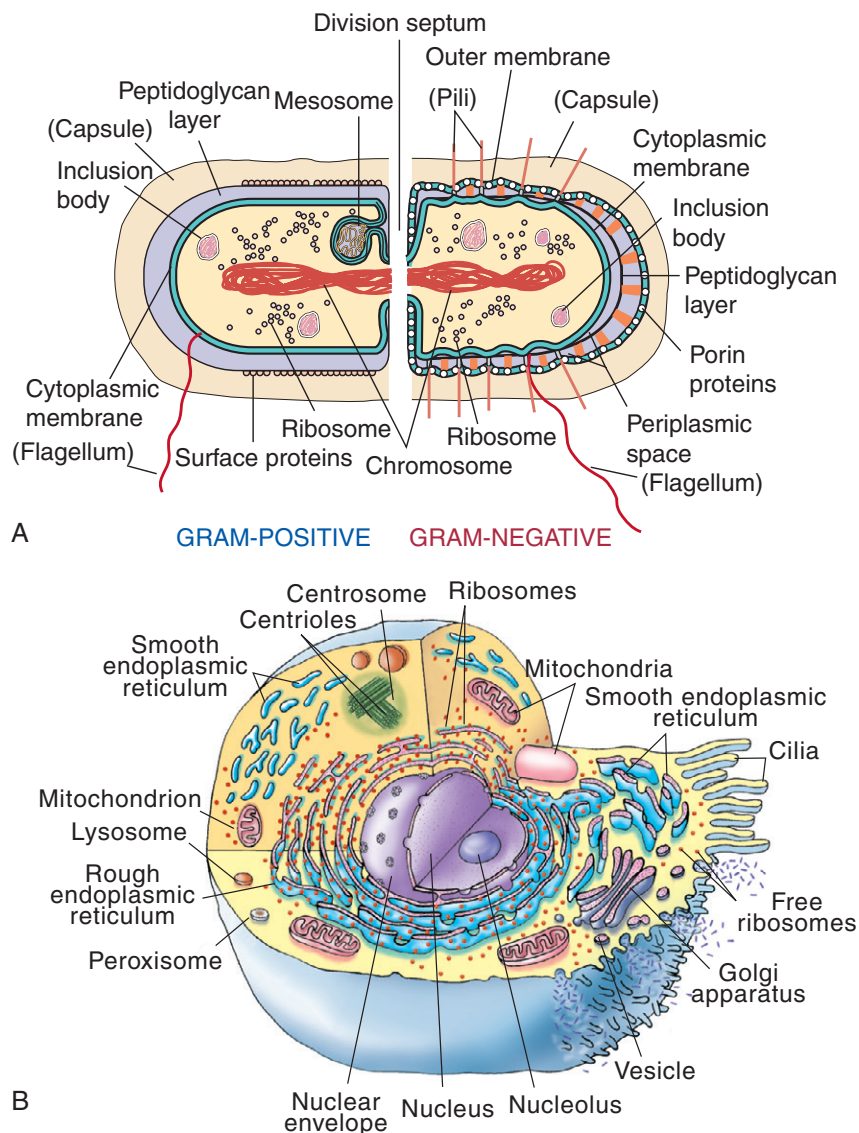


Fig. 1.1 Comparison of prokaryotic and eukaryotic cell organization and structures. A, Prokaryotic gram-positive and gram-negative bacteria. B, Structure of the generalized eukaryotic cell. (A, From Murray, P. R., et al. (2009). *Medical microbiology* [6th ed]. Philadelphia: Mosby; B, from Thibodeau, G. A., & Patton, K. T. (2007). *Anatomy and physiology* [6th ed]. St Louis: Mosby.)

forms), growing as yeast at human temperature (37° C) and as the filamentous form at room temperature (22° C). These fungi are called **dimorphic**. Some systemic fungal diseases in human hosts are caused by dimorphic fungi (see [Chapter 27](#)).

Viruses

Viruses are the smallest infectious particles and cannot be seen under an ordinary light microscope. Often, we can see their effects on cell lines grown in the laboratory, such as inclusions, rounding up of cells, and syncytium (fusion of host cells into multinucleated infected forms), where these characteristics become diagnostic for many viral diseases. These visible changes are called *cytopathic effect*. Viruses are acellular (not composed of cells) and are therefore neither prokaryotic nor eukaryotic. They are distinguished from living cells by the following characteristics:

- Viruses consist of deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), but rarely both. Their genome can be double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), double-stranded RNA (dsRNA), or single-stranded RNA (ssRNA).
- Viruses lack cytoplasmic membranes and are surrounded by a protein coat.
- Viruses are obligate intracellular parasites that cannot self-replicate. They require host cells for replication (increase in number does not involve mitosis, meiosis, or binary fission) and metabolism. Because they lack ribosomes and other metabolites, they “take over” host cell function using the host cell machinery to reproduce. Growth (increase in size) does not occur in viruses.

Viruses are mostly host or host cell specific. For example, human immunodeficiency virus infects T-helper lymphocytes, not muscle cells, in humans, whereas other viruses, such as the rabies virus, can infect dogs, skunks, bats, humans, and other animals. A virus that infects and possibly destroys bacterial cells is known as a **bacteriophage** (Greek *phage*: to eat). Viruses are classified and identified by their genome (DNA or RNA), host range, host disease signs and symptoms, chemical makeup, geographic distribution, the presence or absence of an envelope, their resistance to changes in pH and temperature, their antigenicity (serologic methods), how the virus replicates, and the **virion** (a complete virus outside a cell) size (see [Chapter 29](#)).

Classification/Taxonomy

Taxonomy (Greek *taxes*: arrangement; Greek *nomos*: law) is the orderly classification and grouping of organisms into **taxa** (categories). Taxonomy involves three structured, interrelated categories: classification/taxonomy, nomenclature, and identification. It is based on similarities and differences in **genotype** (genetic makeup of an organism, or combinations of forms of one or a few genes in an organism’s genome) and **phenotype** (observable physical and functional features of an organism expressed by its genotype). Examples of genotypic characteristics include base sequencing of DNA or RNA and DNA base composition ratio to measure the degree of relatedness

of two organisms (see later in this chapter and [Chapter 11](#)). Examples of microbial phenotypic characteristics include macroscopic (colony morphology on media) and microscopic morphology (size, shape, arrangement into groups or chains of cells), staining characteristics (gram-positive or gram-negative), nutritional requirements, physiologic and biochemical characteristics, antigenic markers, and susceptibility or resistance to antimicrobial agents or chemicals. See [Chapters 7, 8, 9, 12, and 13](#) for more detailed information.

Taxa (plural of *taxon*), for example, the levels of classification, are the categories or subsets in taxonomy. The formal levels of bacterial classification in successively smaller taxa or subsets are domain, kingdom, division (or phylum in kingdom Animalia), class, order, family, tribe, genus, species, and subspecies. Below the subspecies level, designations such as serotype or biotype may be given to organisms that share specific minor characteristics. Protists (protozoans) of clinical importance are named similarly to animals; instead of divisions, **phyla** (plural of *phylum*) is used, but the names of the other classifications remain the same. Prokaryotes are placed in the domains Bacteria and **Archaea** (Greek: ancient, origin from the earliest cells), separate from the animals; plants and protists are placed in the domain **Eukarya**. The domains Bacteria and Archaea include unicellular prokaryotic organisms.

Clinical microbiologists traditionally emphasize placement and naming of bacterial species into three (occasionally four or five) categories: the **family** (similar to a human “clan”), a **genus** (equivalent to a human last name), and a **species** (equivalent to a human first name). The plural of genus is *genera*. For example, there are many genera in the family Staphylococcaceae. The proper word for the name of a species is an *epithet*. For example, *Staphylococcus* (genus) *aureus* (species epithet) belongs to the family Staphylococcaceae. Although order and tribe may be useful for the classification of plants and animals, these taxa are not always used for the classification of bacteria. In addition, there are usually different **strains** within a given species of the same species. As an example, there are many different strains of *S. aureus*. If the *S. aureus* isolated from one patient is resistant to penicillin and another *S. aureus* isolate from a different patient is susceptible to penicillin, the two isolates belong to different strains of the same species.

Nomenclature

Nomenclature provides naming assignments for each organism. The following standard rules for denoting bacterial names are used. The family name is capitalized and has an “-aceae” ending (e.g., Micrococcaceae). The genus name is capitalized and followed by the species epithet, which begins with a lowercase letter; both the genus and species should be italicized in print but underlined when written in script (e.g., *Staphylococcus aureus* or Staphylococcus aureus). Often the genus name is abbreviated by use of the first letter (capitalized) of the genus followed by a period and the species epithet (e.g., *S. aureus*). The genus name followed by the word *species* (e.g., *Staphylococcus species*) may be used to refer to the genus as a whole. Species are abbreviated “sp.” (singular) or “spp.” (plural) when the species is not specified. When bacteria are referred to as a group, their names are neither capitalized nor underlined (e.g., staphylococci).

Classification by phenotypic and genotypic characteristics

The traditional method of placing an organism into a particular genus and species is based on the similarity of all members in numerous phenotypic characteristics. In the diagnostic microbiology laboratory, this classification is accomplished by testing each bacterial culture for various metabolic or molecular characteristics and comparing the results with those listed in established tables or databases. In many rapid identification systems, a numeric taxonomy is used in which phenotypic characteristics are assigned a numeric value, and the derived number indicates the genus and species of the bacterium by consulting a database of known organisms.

Epidemiologists constantly seek means of further subdividing bacterial species to follow the spread of bacterial infections. Species are subdivided into subspecies (abbreviated “subsp.”) on the basis of phenotypic differences, serovarieties (abbreviated “serovar”) on the basis of serologic (antigenic) differences, or biovarieties (abbreviated “biovar”) on the basis of biochemical test result differences. Phage typing (based on susceptibility to specific bacterial phages) is also used for this purpose. Current technology allows the analysis of genetic relatedness (DNA and RNA structure and homology) for taxonomic purposes. The analysis of ribosomal RNA (rRNA) gene sequencing is particularly useful for this purpose. The information obtained from these studies resulted in the reclassification of some bacteria.

Classification by cellular type: prokaryotes, eukaryotes, and archaea

Based on cell organization and function, organisms fall into one of three distinct groups: prokaryotes, eukaryotes, or archaea. Taxonomists placed all organisms into three domains that replaced some kingdoms: Bacteria, Archaea, and Eukarya. These three domains are the largest and most inclusive taxa. Each domain is divided into kingdoms on the basis of the similarities of RNA, DNA, and protein sequences. The group prokaryotes includes the domains Archaea and Bacteria (Eubacteria), whereas fungi, algae, protozoa, animals, and plants are eukaryotic in nature and are placed in the domain Eukarya.

The domain Archaea (formerly Archaeobacteria) cell type appears to be more closely related to eukaryotic cells than to prokaryotic cells and is found in microorganisms that grow under extreme environmental conditions. Archaeal cell walls lack peptidoglycan, a major reason they are placed in a domain separate from bacteria. These microbes share some common characteristics with bacteria; they too can stain gram-positive or gram-negative. Gram-positive archaea have a thick wall and stain purple. Gram-negative archaeal cells, in contrast with the typical gram-negative bacterial lipid membrane, have a layer of protein covering the cell wall and stain pink. See the “Gram Stain” section later in this chapter.

The structure of the cell envelope and enzymes of archaea allows them to survive under stressful or extreme (*extremophiles*; lovers of the extreme) conditions. Examples include

halophiles (salt-loving cells) in Utah’s Great Salt Lake, **thermophiles** (heat-loving cells) in hot springs and deep ocean vents, and the anaerobic methanogens that release swamp gas and inhabit the intestinal tracts of animals. Because archaea are not encountered in clinical microbiology, they are not discussed further in this textbook.

In general, the interior organization of eukaryotic cells is more complex than that of prokaryotic cells (see Fig. 1.1). The eukaryotic cell is usually larger and contains membrane-encased organelles (“little organs”) or compartments that serve specific functions, whereas the prokaryotic cell is non-compartmentalized. Various structures are unique to prokaryotic cells (see Fig. 1.1). Differences also exist in the processes of DNA synthesis, protein synthesis, and cell wall synthesis and structure. Table 1.1 compares the major characteristics of eukaryotic and prokaryotic cells.

Pathogenic (disease-causing) **bacteria** are prokaryotic cells that infect eukaryotic hosts. Antimicrobial agents targeting unique prokaryotic structures and metabolism inhibit bacterial growth without harming eukaryotic host cells. This is one reason that pharmaceutical companies have been successful in developing effective antimicrobial agents against bacterial pathogens. However, finding drugs effective against parasites and fungi, which are eukaryotic and resemble their human hosts, and viruses, which use host cells for replication, is less successful. In addition, as a result of genetic changes, bacteria continue to acquire drug resistance.

Comparison of prokaryotic and eukaryotic cell structure

Prokaryotic cell structure

Cytoplasmic structures

Bacteria do not contain a membrane-bound nucleus. Their genome consists of a single circular chromosome. This appears as a diffuse nucleoid or chromatin body (nuclear body) that is attached to a mesosome, a saclike structure in the cell membrane.

Bacterial ribosomes, consisting of RNA and protein, are found free in the cytoplasm and attached to the cytoplasmic membrane. They are the site of protein synthesis. They are 70S in size and dissociate into two subunits: 50S and 30S (see Table 1.1). The *S* stands for *Svedberg units*, which refer to sedimentation rates (unit of time) during high-speed centrifugation. The Svedberg unit is named for Theodor Svedberg, Nobel Prize winner and inventor of the ultracentrifuge. Larger particles have higher *S* values. The *S* value is *not additive*. When the previously mentioned two subunits 50S and 30S bind together, there is a loss of surface area, and the two subunits produce a complex 70S in size. The same occurs in the eukaryotic cell, where the two subunits 60S and 40S combine to form an 80S complex.

Stained bacteria sometimes reveal the presence of cytoplasmic granules. These granules are storage deposits and may consist of polysaccharides such as glycogen, lipids such as poly β -hydroxybutyrate, or polyphosphates. These granules are sometimes referred to as *metachromatic granules* and can be visualized with the methylene blue stain.

Certain genera, such as *Bacillus* and *Clostridium*, produce endospores in response to harsh environmental conditions. Endospores are small, dormant (inactive), asexual spores that develop inside the bacterial cell as a means of survival, not reproduction. Their thick protein coat makes them highly resistant to chemical agents, temperature change, starvation, dehydration, ultraviolet and gamma radiation, and desiccation. Under harsh conditions, each vegetative cell (active, capable of growing and dividing) produces one endospore (inactive) that later germinates under favorable environmental conditions into one vegetative cell. Endospores should not be confused with the reproductive spores of fungi (see Chapter 27).

Spores appear as highly refractile bodies in the cell and are visualized with Gram stain as unstained areas in a cell because of their thick protein coat. With the *Schaeffer-Fulton* stain, the most commonly used endospore stain, endospores appear green (malachite green is used as the primary stain), and the bacterial cells are red (safranin is the counterstain). The size, shape, and interior location of the spore, for example, at one end (terminal), subterminal, or central, can be used as identifying characteristics. For instance, the terminal spore of *Clostridium tetani*, the etiologic (causative) agent of tetanus, causes swelling of the thin rod-shaped cell giving the organism a characteristic tennis racquet-shaped or lollipop-shaped appearance.

Cell envelope structures

The cell envelope consists of the membrane and structures surrounding the cytoplasm. In bacteria, these are the plasma membrane and the cell wall. Some species also produce capsules and slime layers.

Plasma (Cell) membrane

The plasma membrane is a phospholipid bilayer with embedded proteins that surrounds the cytoplasm. The prokaryotic plasma membrane is made of phospholipids and proteins and (except for those in the family *Mycoplasmataceae*, which

do contain sterols) does not contain sterols. This is in contrast with eukaryotic plasma membranes, which contain sterols. The plasma membrane acts as an osmotic barrier (prokaryotes have a high osmotic pressure inside the cell) and is the location of the electron transport chain, where energy is generated. The general functions of the prokaryotic plasma membrane are identical to functions in eukaryotes, except for energy generation (Fig. 1.2).

Cell wall

The cell wall of prokaryotes is a rigid structure that maintains the shape of the cell and prevents bursting of the cell from the high osmotic pressure inside it. Cell walls in bacteria have been traditionally categorized according to their staining characteristics. The two major types of cell walls are **gram-positive** and **gram-negative** (see Fig. 1.1A). They can be differentiated by the Gram stain.

Gram-positive cell wall

The gram-positive cell wall is composed of a very thick peptidoglycan (murein) layer. The peptidoglycan layer consists of glycan (a polysaccharide) and chains of alternating *N*-acetyl-D-glucosamine (NAG) and *N*-acetyl-D-muramic acid (NAM) (Fig. 1.3). Short peptides, each consisting of four amino acid residues, are attached to a carboxyl group on each NAM residue. The chains are then cross-linked to form a thick network via a peptide bridge (differing in number of peptides) connected to the tetrapeptides on the NAM. Because the peptidoglycan layer is the principal component of the gram-positive cell wall, antimicrobial agents that target gram-positive organisms (e.g., penicillins) are effective by preventing synthesis of peptidoglycan. Gram-negative bacteria, which have a different cell wall structure, are less affected by these agents.

Other components of the gram-positive cell wall that penetrate to the exterior of the cell are teichoic acid, anchored to the peptidoglycan, and lipoteichoic acid, anchored to the plasma membrane. These two components are unique to the gram-positive cell wall. Other antigenic polysaccharides may be present on the surface of the peptidoglycan layer.

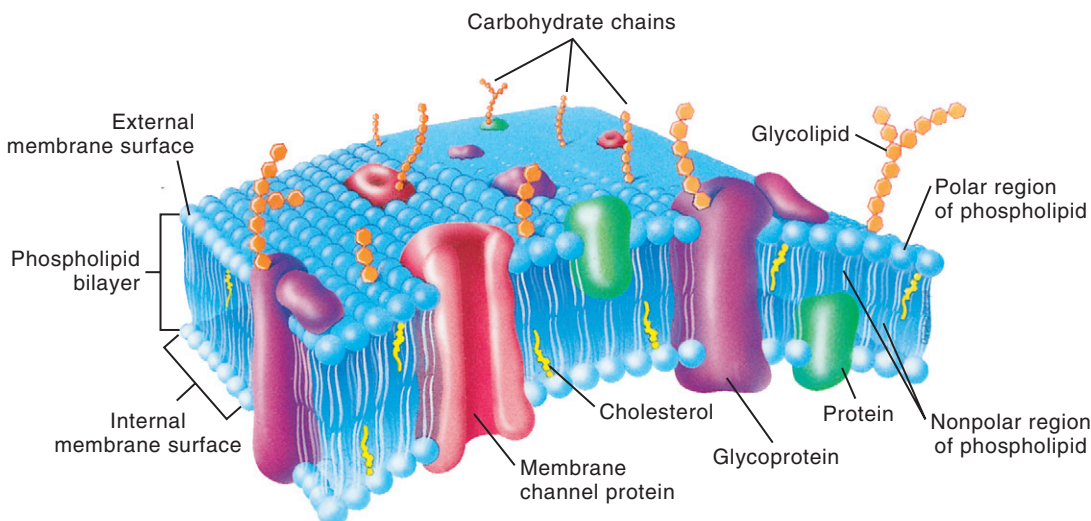


Fig. 1.2 Structure of the plasma membrane. (From Thibodeau, G. A., & Patton, K. T. (2007). *Anatomy and physiology* [6th ed]. St Louis: Mosby.)

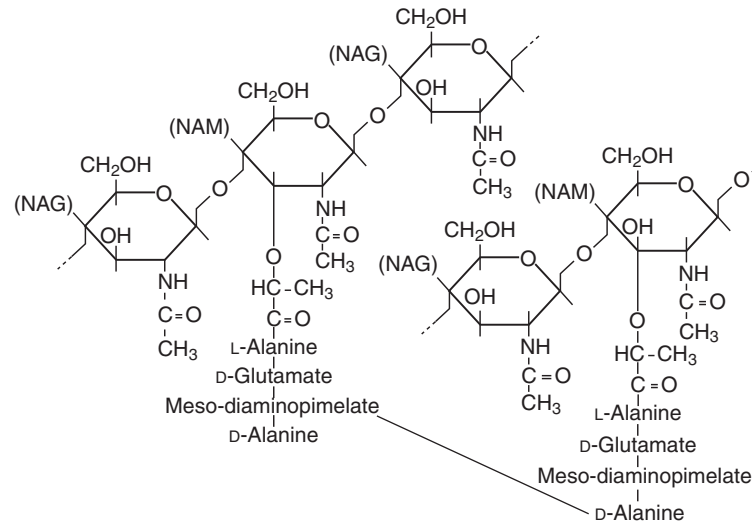


Fig. 1.3 The structure of the peptidoglycan layer in the cell wall of *Escherichia coli*. The amino acids in the cross-linking tetrapeptides may differ among species. NAG, N-acetyl-D-glucosamine; NAM, N-acetyl-D-muramic acid. (From Neidhardt, F. C., et al. (1990). *Physiology of the bacterial cell: a molecular approach*. Sunderland, MA: Sinauer Associates.)

Gram-negative cell wall

The cell wall of gram-negative bacteria comprises two layers: the inner peptidoglycan layer, which is much thinner than in gram-positive cell walls, and an additional outer membrane unique to the gram-negative cell wall. The outer membrane, sometimes called an *envelope*, contains proteins, phospholipids, and lipopolysaccharide (LPS). LPS contains three regions: an antigenic O-specific polysaccharide, a core polysaccharide, and an inner lipid A (also called *endotoxin*). The lipid A moiety is responsible for producing fever and shock in animals infected with gram-negative bacteria. The toxicity varies among different bacterial species. The outer membrane has the following functions:

- It acts as a barrier to hydrophobic compounds and harmful substances.
- It acts as a sieve, allowing water-soluble molecules to enter through protein-lined channels called *porins*.
- It provides attachment sites that enhance attachment to host cells.

Between the outer membrane and the inner membrane and encompassing the thin peptidoglycan layer is an area referred to as the *periplasmic space*. Within the periplasmic space is a gel-like matrix containing nutrient-binding proteins and degradative and detoxifying enzymes. The periplasmic space is absent in gram-positive bacteria.

Acid-fast cell wall

Certain genera, such as *Mycobacterium* and *Nocardia*, have a gram-positive cell wall structure and are classified as gram-positive bacteria. However, because they contain a waxy layer of glycolipids and fatty acids (mycolic acid) bound to the exterior of the cell wall, they stain poorly with the Gram stain. More than 60% of the cell wall is lipid, and the major lipid component is mycolic acid, a strong hydrophobic molecule that forms a lipid shell around the organism and affects its permeability. This excludes water-soluble

Case check 1.1

The differential ability of the Gram stain makes it useful in classifying a bacterium as gram-positive or gram-negative. As in the Case in Point, correct interpretation and assessment of the Gram-stained smear results are critical in the presumptive identification of the organism present. See also Procedure 12 in [Appendix D on Evolve](#). The use of quality control organisms with known Gram stain reactions ensures that the Gram stain procedure is performed correctly. Bacteria with a thick peptidoglycan layer in the cell wall containing teichoic acid cross-linkages retain the crystal violet-iodine dye complex after decolorization and appear deep blue. They are gram-positive (e.g., *S. aureus*). Bacteria that possess an outer membrane, a lipid bilayer, and a thin layer of peptidoglycan do not retain the dye complex when exposed to a decolorizer. They are gram-negative (e.g., *E. coli*). The alcohol-acetone decolorizer damages the outer membrane of gram-negative bacteria and allows the stain complex to wash out. All unstained elements, such as gram-negative bacteria and products of inflammation, are subsequently counterstained red by the dye safranin. Older, dying, or dead gram-positive bacterial cells, having lost the integrity of the thick peptidoglycan layer, are not able to retain the crystal violet-iodine complex and will also appear red.

dyes and makes *Mycobacterium* spp. difficult to stain with the Gram stain. Mycobacteria are best seen with an acid-fast stain, in which carbol fuchsin is the primary stain, followed by an acid-alcohol decolorizer. See "Acid-fast Stains" later in this chapter and also Procedure 12 in Appendix D on Evolve.

Absence of cell wall

Prokaryotes that belong to the genera *Acholeplasma*, *Mycoplasma*, and *Ureaplasma* are unique in that they lack a cell wall and contain sterols in their plasma membranes. Because they lack the rigidity of the cell wall, they are seen in various shapes microscopically referred to as being **pleomorphic**. Some gram-positive and gram-negative cells can lose their cell walls and grow as L-forms in media supplemented with serum or sugar to prevent osmotic rupture of the cell membrane.

Surface polymers

Various pathogenic bacteria produce a discrete organized covering termed a **capsule**. Capsules are usually made of polysaccharide polymers, although they may also be made of polypeptides. Capsules act as virulence factors in helping the pathogen evade phagocytosis. During identification of certain bacteria by serologic typing, capsules sometimes must be removed to detect the somatic (cell wall) antigens present underneath them. Capsule removal is accomplished by boiling a suspension of the microorganism. *Salmonella* Typhi must have its capsular (Vi) antigen removed for the laboratory scientist to observe agglutination with *Salmonella* somatic (O) antisera. The capsule does not ordinarily stain with use of common laboratory stains, such as Gram stain or India ink. Instead, it appears as a clear area (“halo like”) between or surrounding the stained organism and the stained amorphous background material in a direct smear from a clinical specimen.

Slime layers or a *glycocalyx* are similar to capsules but are more diffuse layers surrounding the cell. They also are made of polysaccharides and serve either to inhibit phagocytosis or, in some cases, to aid in adherence to host tissue or synthetic implants. Glycocalyx production can be the first step in the formation of a biofilm (see [Chapter 31](#)).

Case check 1.2

The most common mechanism for evading phagocytosis used by many microorganisms is having a surface polysaccharide capsule. Microorganisms possessing a capsule are generally highly virulent, as in the Case in Point, until removal of the capsule, at which point virulence becomes extremely low. Encapsulated strains of *Streptococcus pneumoniae* and *Haemophilus influenzae* are associated with highly invasive infections and are known to be more virulent than nonencapsulated strains. See also the discussion on the ability to resist phagocytosis in [Chapter 2](#). Antibodies produced against the capsule often lead to phagocytosis and immunity against that bacterial strain.

Cell appendages

Flagella are exterior protein filaments that rotate and cause bacteria to be motile. Bacterial species differ in their possession of flagella from none (nonmotile) to many ([Fig. 1.4](#)). Flagella that extend from one end of the bacterial cell are polar. Polar flagella may occur singly at one end (*monotrichous*) or both ends (*amphitrichous*) or multiply in tufts at one end (*lophotrichous*). Flagella that occur all around the bacterium are *peritrichous*. The number and arrangement of flagella are sometimes used for identification purposes. Flagella can be visualized microscopically with special flagellum stains.

Pili (plural of *pilus*) and **fimbriae** (plural of *fimbria*) are nonflagellar, proteinaceous, hairlike appendages extending a short distance from the surface of some bacterial cells that adhere cells to one another and to host cells. *Conjugation pili* are protein tubes that connect two bacterial cells and mediate DNA exchange.

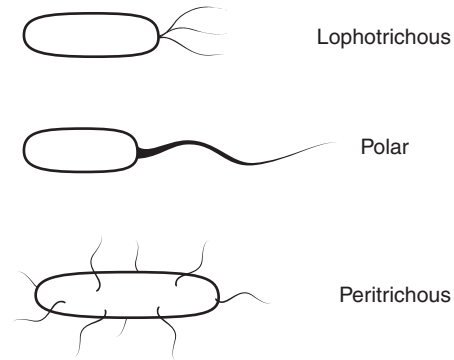


Fig. 1.4 Three flagellar arrangements found in bacteria. Other variations can occur.

Eukaryotic cell structure

The following structures are associated with eukaryotic cells (see [Table 1.1](#) and [Fig. 1.1](#)). In the diagnostic microbiology laboratory, medically important fungi and parasites have eukaryotic cells.

Cytoplasmic structures

The nucleus of the eukaryotic cell contains the DNA of the cell in the form of discrete chromosomes—structures in the nucleus that carry genetic information (genes)—contained within a nuclear membrane. Chromosomes are covered with basic proteins called *histones*. The number of chromosomes in the nucleus differs according to the particular organism. A rounded, refractile body called a *nucleolus* is also located within the nucleus. The nucleolus is the site of rRNA synthesis. The nucleus is bounded by a bilayered lipoprotein nuclear membrane.

The ER is a system of membranes found throughout the cytoplasm. It occurs in two forms. The “rough” ER is covered with ribosomes and is the site of protein synthesis. The ribosomes give the ER the rough appearance. The smooth ER does not have ribosomes on the outer surface of its membrane, hence its smooth appearance. Smooth ER does not synthesize proteins, but it does synthesize phospholipids. The major function of the Golgi apparatus or complex is to modify and package proteins and lipids sent to it by the ER.

Eukaryotic ribosomes, where protein synthesis occurs, are 80S in size and dissociate into two subunits: 60S and 40S. They are attached to the rough ER. Eukaryotic cells contain several membrane-enclosed organelles. Mitochondria are the main site of energy production. They contain DNA and the electron transport system that produces energy in the form of adenosine triphosphate (ATP). Lysosomes contain hydrolytic enzymes for degradation of macromolecules and microorganisms within the cell. Peroxisomes contain protective enzymes that break down hydrogen peroxide and other peroxides generated within the cell. Chloroplasts, found in plant cells, are the sites of photosynthesis. Chloroplasts are the sites where light energy is converted into chemical energy (ATP). Photosynthesis produces oxygen from carbon dioxide and water. Fungi are not plants and have no chloroplasts.

Cell envelope structures

Plasma membrane

The plasma membrane (see Fig. 1.2) is a phospholipid bilayer with embedded proteins that envelops the cytoplasm and regulates transport of macromolecules into and out of the cell. A substantial amount of cholesterol is found in the plasma membrane of animals. Cholesterol has a stabilizing effect and helps keep the membrane fluid. The polar heads of the phospholipids are hydrophilic (water loving) and lie on the intracellular and extracellular sides of the membrane; their nonpolar tails are hydrophobic (water hating) and avoid water by lining up in the center of the plasma membrane “tail to tail.” This type of hydrophobic makeup of the interior of the plasma membrane makes it potentially impermeable to water-soluble molecules. Proteins, embedded in the membrane, perform several important functions. They can act as enzymes, hormone receptors, pore channels, and carriers.

Cell wall

The function of a cell wall is to provide rigidity and strength to the exterior of the cell. Most eukaryotic cells do not have cell walls. However, fungi have cell walls principally made of polysaccharides, such as chitin, mannan, and glucan. Chitin is a distinct component of fungal cell walls.

Motility organelles

Cilia are short projections (3 to 10 μm), usually numerous, that extend from the cell surface and are used for locomotion. They are found in certain protozoa and in ciliated epithelial cells of the respiratory tract. Flagella are longer projections (>150 μm) used for locomotion by protozoa and animal cells such as spermatozoa.

Bacterial morphology

Microscopic shapes

The largest bacterium known, *Thiomargarita namibiensis*, is found in ocean sediment and generally has a diameter of 0.1 to 0.3 mm. Most bacteria range in size from 0.4 to 2 μm . They occur in three basic shapes (Fig. 1.5):

- Cocci (spherical)
- Bacilli (rod-shaped)
- Spirochetes (spiral)

Individual bacterial cells may form characteristic groupings. Cocci (plural of *coccus*) can occur singly, in pairs (diplococci), in chains (streptococci), or in clusters (staphylococci). Bacilli (plural of *bacillus*) vary greatly in size and length from very short coccobacilli to long filamentous rods. The ends may be square or rounded. Bacilli with tapered, pointed ends are termed **fusiform**; these cells are sometimes curved. When a species differs in size and shape within a pure culture, the bacterium is termed *pleomorphic*. Bacilli can occur as single rods or in chains or may align themselves side by side (palisades) or be branching. Spirochetes vary in length and in the number of helical turns; not all helical bacteria are called *spirochetes*.

Microscopic Morphology of Bacteria

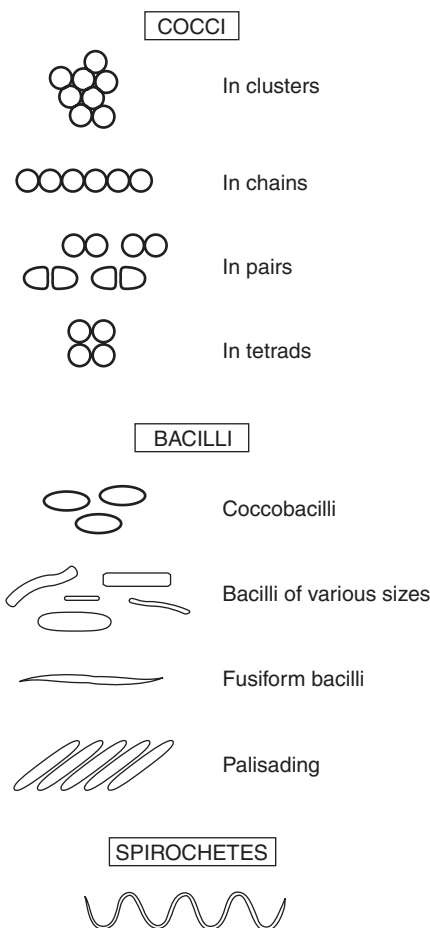


Fig. 1.5 The microscopic shapes and arrangements of bacteria.

Common stains used for microscopic visualization

Stains that impart color or fluorescence are needed to visualize microscopically bacteria. The microscopic staining characteristics (i.e., shapes and groupings) are used in the classification and identification of microorganisms (Fig. 1.6).

Gram stain

The Gram stain is the most commonly used stain in the clinical microbiology laboratory. It places bacteria into one of two groups: gram-positive (blue to purple) or gram-negative (pink or red; see Fig. 1.6A–B). Some organisms are gram-variable or do not stain at all. As mentioned previously, the cell wall structure determines the Gram-staining characteristics. The Gram stain consists of gentle heat fixing (methyl alcohol may be used instead for fixation) of the smear and the addition of four sequential components: crystal violet (the primary stain, 1 minute), iodine (the mordant or fixative, 1 minute), alcohol or an alcohol-acetone solution (the decolorizer, a quick rinse), and safranin (the counterstain, 30 seconds). The time frames listed are not exact and vary with the organism; rinsing with water between each

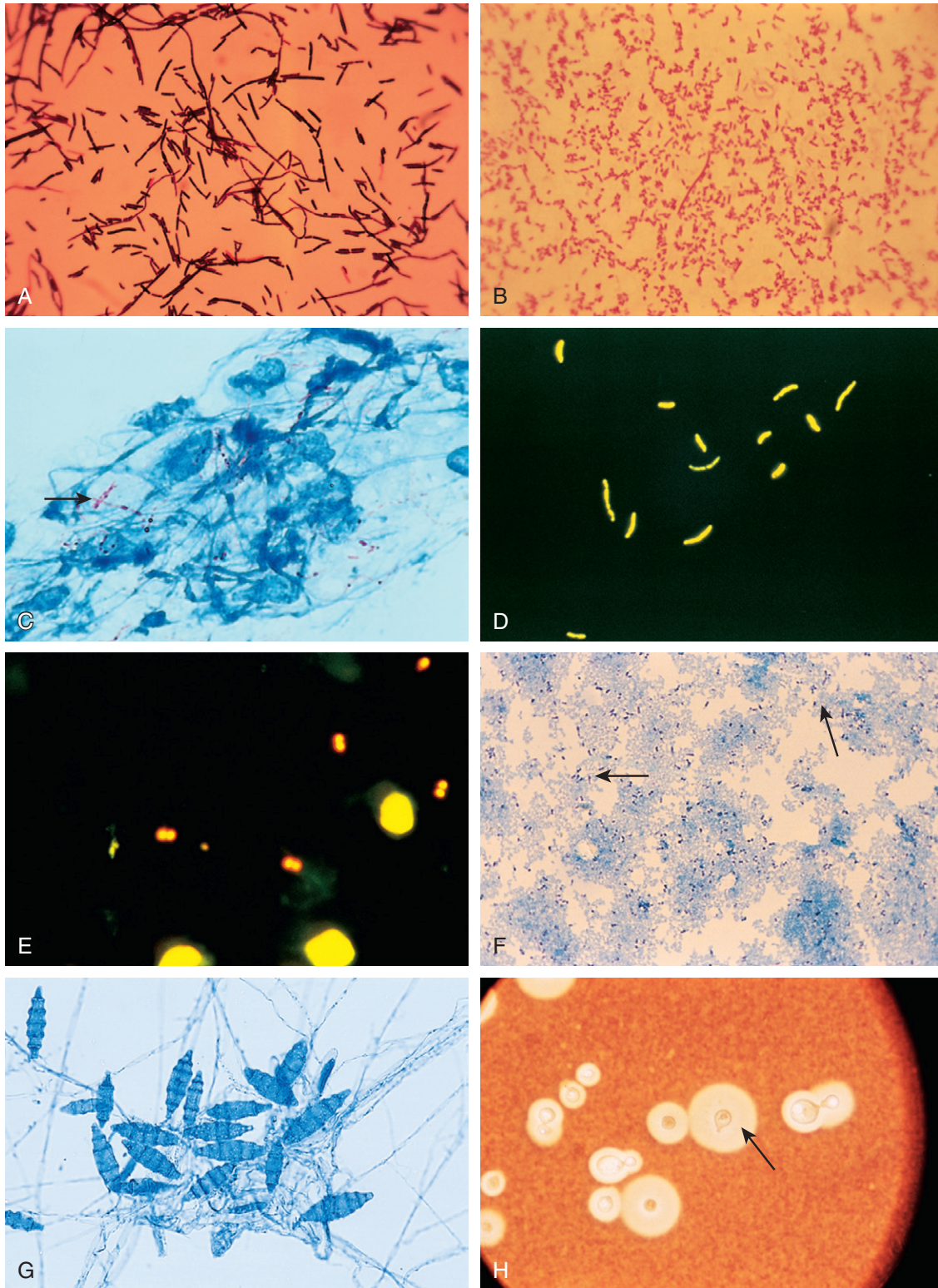


Fig. 1.6 **A**, Gram stain of *Lactobacillus* species illustrating gram-positive bacilli, singly and in chains. A few gram-negative-staining bacilli are also present. **B**, Gram stain of *Escherichia coli* illustrating short gram-negative bacilli. **C**, Acid-fast stain, carbol fuchsin–based. Sputum smear demonstrating the presence of acid-fast *Mycobacterium* species (arrow) stained by the Kinyoun or Ziehl-Neelsen carbol fuchsin method. **D**, Acid-fast stain, fluorochrome–based. *Mycobacterium* species stained with the acid-fast fluorescent auramine-rhodamine stain. This stain is useful for screening for the presence of acid-fast bacteria in clinical specimens. **E**, Acridine orange stain. Fluorescent stain demonstrating the presence of staphylococci in a blood culture broth. This stain is useful for detecting bacteria in situations in which debris may mask the bacteria. **F**, Methylene blue stain. Methylene blue stain demonstrating the typical morphology of *Corynebacterium diphtheriae* (arrows). **G**, Lactophenol cotton blue stain. Lactophenol cotton blue–stained slide of macroconidia and hyphae of the fungal dermatophyte *Microsporum gypseum*. **H**, India ink. An India ink wet mount of the yeast *Cryptococcus neoformans* demonstrating the presence of a capsule (arrow). (A and B, Courtesy Dr. Andrew G. Smith, Baltimore, MD; D, courtesy Clinical Microbiology Audiovisual Study Units, Health and Education Resources, Inc., Bethesda, MD; E, courtesy Dr. John E. Peters, Baltimore, MD; and H, courtesy Dr. Andrew G. Smith, Baltimore, MD.)

step is important. The bacteria are initially stained purple by the crystal violet that is bound to the cell wall with the aid of iodine. When the decolorizer is applied to bacteria with a gram-negative cell wall, the crystal violet washes out of the cells, which then take up the pink counterstain, safranin. For this reason, gram-negative bacteria appear pink under the light microscope. Bacteria with a gram-positive cell wall retain the primary crystal violet stain during the decolorizing treatment and appear purple. Cells in a direct smear from a patient specimen, such as epithelial cells, white blood cells, red blood cells, and amorphous background material, should appear pink if the Gram stain procedure was performed correctly.

Case check 1.3

Review of quality control slides is important in detecting errors in the performance of the Gram stain procedure and in interpreting results. As illustrated in the Case in Point, the gram-positive control organism, *S. aureus*, stained gram-positive, which is an acceptable result. However, the gram-negative control organism, *E. coli*, also appeared gram-positive, which is an unacceptable result and indicative of an error in performing the Gram stain procedure. When such an error occurs, the results cannot be reported until the discrepancy is resolved, and the procedure is repeated with acceptable quality control results.

Acid-fast stains

Acid-fast stains are used to stain bacteria that have a high lipid (mycolic acid) and wax content in their cell walls and do not stain well with traditional bacterial stains. Carbol fuchsin, a red dye, is used as the primary stain (see Fig. 1.6C). Mycolic acid makes the bacterial cell resistant to acid-alcohol decolorization and hence the bacteria retain the primary stain. Mycobacteria, because they retain the stain, are designated acid-fast bacteria. The cell wall is treated to allow penetration of the dye either by heat (Ziehl-Neelsen method) or by a detergent (Kinyoun method). Acidified alcohol is used as a decolorizer, and methylene blue, or sometimes malachite green, is the counterstain. Acid-fast bacteria retain the primary stain and are pink. Bacteria that are not acid fast are blue or green depending on the counter stain. Two other gram-positive genera, *Nocardia* and *Rhodococcus*, may stain acid-fast by a modified method that utilizes a weaker decolorizer. They are generally considered partially acid-fast when stained with a modified acid-fast stain. In addition, the nocardiae give a darker blue appearance on Gram stain compared with the faint blue for mycobacteria. Acid-fast staining is also used to identify *Saccharomyces*, a yeast, and coccidian parasites, such as *Cystoisospora belli* (formerly known as *Isospora belli*), *Cryptosporidium*, and other coccidia like bodies.

A fluorochrome (i.e., fluorescent) stain, auramine-rhodamine, is also used to screen specimens for acid-fast bacteria (see Fig. 1.6D). This stain is selective for the cell wall of acid-fast bacteria. Acid-fast bacteria appear yellow or orange under a fluorescent microscope, making them easier to find and the staining method more sensitive than carbol fuchsin-based stains. Non-acid-fast bacteria are unstained.

Acridine orange

Acridine orange is a fluorochrome dye that stains gram-positive and gram-negative bacteria, living or dead. It binds to the nucleic acid of the cell and fluoresces a bright orange when a fluorescent microscope is used. Acridine orange is used to locate bacteria in blood cultures and other specimens in which discerning bacteria might otherwise be difficult (see Fig. 1.6E). Acridine orange will stain eukaryotic cells as well.

Calcofluor white

Calcofluor white is a fluorochrome that binds to chitin in fungal cell walls. It fluoresces a bright apple-green or blue-white, allowing visualization of fungal structures with a fluorescent microscope. Calcofluor white was the original “bluing” used in high-volume laundries to whiten yellow-appearing white cotton and other fabrics. See also Procedure 5 in Appendix D on Evolve.

Methylene blue

Methylene blue traditionally has been used to stain *Corynebacterium diphtheriae* for observation of metachromatic granules (see Fig. 1.6F). It is also used as a counterstain in acid-fast staining procedures. It is sometimes used as a simple stain to detect white blood cells, such as in stool samples.

Lactophenol cotton blue

Lactophenol cotton blue is used to stain the cell walls of medically important fungi grown in slide cultures (see Fig. 1.6G). It utilizes the blue dye aniline.

India ink

India ink and nigrosin are negative stains used to visualize capsules surrounding certain yeasts, such as the yeast *Cryptococcus* spp. (see Fig. 1.6H). The fine ink particles are excluded from the capsule, leaving a dark background and a clear capsule surrounding the yeast cells.

Endospore stain

The Schaefer-Fulton spore stain is used to stain bacterial spores. The primary stain, malachite green, is applied (flooded) to a heat-fixed smear and heated to steaming for about 5 minutes. Then the preparation is washed for about 30 seconds to remove the primary stain. Next, the counterstain safranin is applied to the smear. The endospores appear green within pink- or red-appearing bacterial cells.

Microbial growth and nutrition

All bacteria have three major nutritional needs for growth:

- A carbon source for making cellular constituents. Carbon represents 50% of the dry weight of a bacterium.
- A nitrogen source for making proteins and nucleic acids. Nitrogen makes up 14% of the dry weight.
- An energy source (ATP) for performing cellular functions.

Smaller amounts of molecules, such as phosphate for nucleic acids and phospholipids of cell membranes and sulfur for protein synthesis, make up an additional 4% of the weight. Various metals and ions for enzymatic activity must also be present in trace amounts. Important mineral ions, such as Na^+ , K^+ , Cl^- , Fe^{2+} , and Ca^{2+} , are also required. Although the basic building blocks required for growth are the same for all cells, bacteria differ widely in their ability to use different sources of these molecules.

Nutritional requirements for growth

Bacteria are classified into two groups according to how they meet their nutritional needs. The **autotrophs** (lithotrophs) are able to grow simply, using carbon dioxide as the sole source of carbon, in addition to the required water and inorganic salts. Autotrophs obtain energy either photosynthetically (phototrophs) or by oxidation of inorganic compounds (chemolithotrophs). Autotrophs occur in environmental milieus.

The second group of bacteria, the **heterotrophs**, require more complex substances for growth. These bacteria require an organic source of carbon, such as glucose, and obtain energy by oxidizing or fermenting organic substances. Often, the same substance (e.g., glucose) is used as both the carbon source and the energy source.

All bacteria that inhabit the human body are heterotrophs. However, nutritional needs differ greatly within this group. Bacteria such as *E. coli* and *Pseudomonas aeruginosa* can use a wide variety of organic compounds as carbon sources and grow on most simple laboratory media. Other pathogenic bacteria, such as *H. influenzae* and the anaerobes, are fastidious, requiring additional metabolites such as vitamins, purines, pyrimidines, and hemoglobin supplied in the growth medium. Some pathogenic bacteria, such as *Chlamydia* spp., cannot be cultured on laboratory media and must be grown in cell culture or detected by other means.

Types of growth media

Various types of growth media are used in the diagnostic microbiology laboratory to cultivate and recover bacteria from clinical samples. Culture media are categorized according to their function and ability to support growth. Those

that contain nutrients that support the growth of most non-fastidious organisms are called **nonselective** or **nutritive** type of media. Examples of nutrient media include trypticase soy agar or broth and nutrient agar. A growth medium that contains added growth factors, such as blood, vitamins, and yeast extract, is referred to as an **enriched** medium (e.g., sheep blood agar and chocolate agar). The added factors allow fastidious organisms to grow.

Media that contain additives such as dyes, bile salts, alcohols, acids, and antimicrobial agents that inhibit the growth of some bacteria but allow others to grow are called **selective media**. MacConkey (MAC) agar is an example of a selective medium for gram-negative bacteria. It contains bile salts and crystal violet that inhibit gram-positive bacteria but allow the growth of gram-negative bacteria. Columbia agar with colistin and nalidixic acid, alternatively, is selective for gram-positive organisms by inhibiting the growth of gram-negative bacteria. Media that contain ingredients that allow visualization of metabolic differences between groups or species of bacteria are called **differential media**. MAC agar, a selective medium, is also a differential medium because it distinguishes between lactose fermenters (pink colonies) and nonlactose fermenters (clear colonies) (Fig. 1.7A–B). Sheep blood agar, an enriched, nonselective type of medium, is also differential because it distinguishes between hemolytic and nonhemolytic organisms. Fig. 1.8 shows a sheep blood agar plate containing beta-hemolytic colonies. Therefore some media can be selective and differential (e.g., MAC agar) or nonselective and differential (e.g., sheep blood agar).

Broth media, such as thioglycollate broth, can be used to supplement agar media to recover small numbers of organisms that may be present in a clinical sample. **Enrichment broth** is designed to encourage the growth of small numbers of a particular organism while suppressing other bacteria that might be present in the specimen. An example is Lim broth (Todd Hewitt with colistin and nalidixic acid) used to enhance the growth of group B streptococci. Enrichment broths are incubated for a specific time and then must be subcultured to solid media to isolate the organism of interest.

When a delay between collection of the specimen and culturing is necessary, a **transport medium** is used. A transport medium is a holding medium designed to preserve the

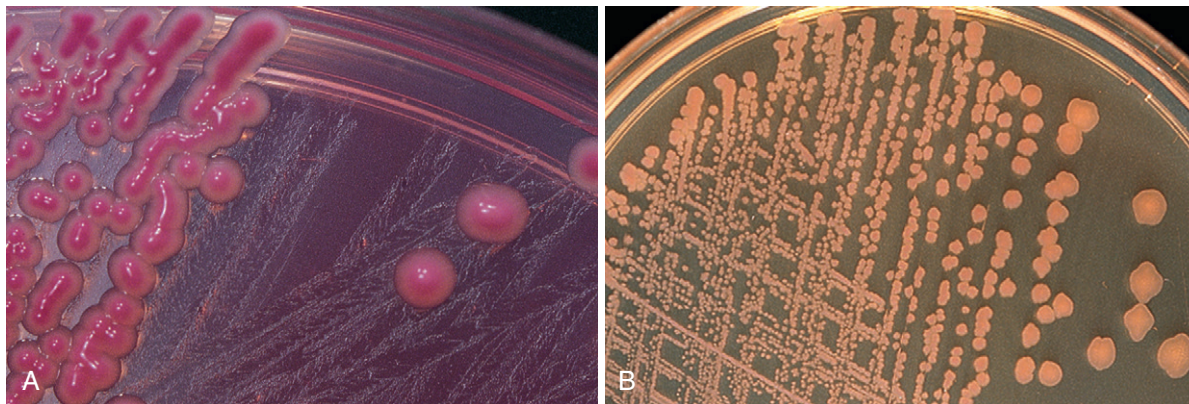


Fig. 1.7 **A**, Lactose-fermenting, gram-negative rods producing pink colonies on MacConkey (MAC) agar. **B**, Nonlactose fermenting, gram-negative rods producing colorless colonies on MAC agar.

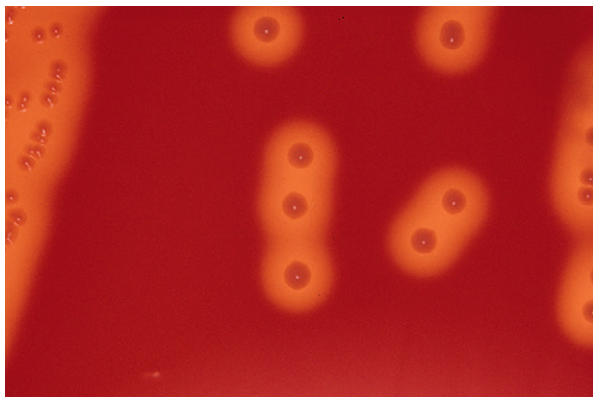


Fig. 1.8 Blood agar plate showing beta-hemolytic colonies.

viability of microorganisms in the specimen but not allow multiplication. Stuart broth and Amies and Cary-Blair transport media are commonly used examples.

Environmental factors influencing growth

The following three important environmental factors influence the growth rate of bacteria and must be considered when bacteria are cultured in the laboratory:

- pH
- Temperature
- Gaseous composition of the atmosphere

Most pathogenic bacteria grow best at a neutral pH. Diagnostic laboratory media for bacteria are usually adjusted to a final pH between 7.0 and 7.5. Temperature influences the rate of growth of a bacterial culture. Microorganisms are categorized according to their optimal temperature for growth. Bacteria that grow best at cold temperatures are called **psychrophiles** (optimal growth at 10° to 20° C). Bacteria that grow optimally at moderate temperatures are called **mesophiles** (optimal growth at 20° to 40° C). Bacteria that grow best at high temperatures are called **thermophiles** (optimal growth at 50° to 60° C). Psychrophiles and thermophiles are found environmentally in places such as the Arctic seas and hot springs, respectively. Most bacteria that have adapted to humans are mesophiles, which grow best near the human core body temperature of 37° C. Diagnostic laboratories routinely incubate cultures for bacterial growth at 35° C. However, some pathogenic species prefer a lower temperature for growth; when these organisms are suspected, the media from these specimens are incubated at a lower temperature. Fungal cultures are incubated at 30° C. The ability to grow at room temperature (22° C) or at an elevated temperature (42° C) is used as an identification characteristic for some bacteria.

Bacteria that infect humans differ in their atmospheric requirements for growth. In the diagnostic microbiology laboratory, bacterial atmospheric growth requirements are optimized to ensure recovery and isolation of pathogenic organisms from clinical samples. Microorganisms that require oxygen for growth are called **obligate aerobes**, and those that cannot grow in the presence of oxygen are

obligate or **strict anaerobes**. Obligate anaerobes must be grown in an atmosphere either devoid of oxygen or with significantly reduced oxygen content. **Aerotolerant anaerobes** can survive in the presence of oxygen but grow poorly and do not use oxygen in metabolism; **facultative anaerobes** can grow either with or without oxygen. If oxygen is present, the facultatively anaerobic bacteria will utilize it via aerobic respiration and grow faster than without oxygen. Facultative anaerobes like *E. coli* are routinely cultured in an aerobic atmosphere because aerobic culture is easier and less expensive than anaerobic culture, and the bacteria grow more rapidly.

Microaerophilic bacteria require a reduced level of oxygen to grow. An example of a pathogenic microaerophile is *Campylobacter jejuni*, which requires 5% to 6% oxygen. The atmosphere required by microaerophiles can be generated in culture jars or pouches with a commercially available microaerophilic atmosphere-generating system.

Capnophilic organisms are those that require an atmosphere enriched with carbon dioxide (5% to 10%); an example is *Neisseria gonorrhoeae*. The concentration of carbon dioxide in ambient air is about 400 parts per million. Air contains approximately 21% oxygen. When the carbon dioxide content of an aerobic incubator is increased to 10%, the oxygen content of the incubator is decreased to approximately 18%. Obligate aerobes must have oxygen to grow; incubation in air or an aerobic incubator with 10% carbon dioxide satisfies their oxygen requirement. Because many bacteria grow better in the presence of increased carbon dioxide, diagnostic microbiology laboratories often maintain their aerobic incubators at a 5% to 10% carbon dioxide level, in what is commonly referred to as a *carbon dioxide incubator*.

Bacterial growth

Generation time

Bacteria replicate by binary fission, with one cell dividing into two cells. The time required for one cell to divide into two cells is called the *generation time* or *doubling time*. The generation time of a bacterium in culture can be 20 minutes for a fast-growing bacterium such as *E. coli* or 24 hours for a slow-growing bacterium such as *Mycobacterium tuberculosis*.

Growth curve

If bacteria are in a growth state with enough nutrients and no toxic products present, the increase in bacterial numbers is proportional to the increase in other bacterial properties, such as mass, protein content, and nucleic acid content. Measurement of any of these properties can be used as an indication of bacterial growth. When the growth of a bacterial culture (number of live cells) is plotted over time, the resulting growth curve shows four phases of growth: (1) a lag phase in which bacteria are preparing to divide; (2) an exponential phase in which bacterial numbers increase logarithmically; (3) a stationary phase in which nutrients are becoming limited and the numbers of bacteria remain constant (although viability may decrease); and (4) a death phase in which the number of nonviable bacterial cells exceeds the

number of viable cells. An example of such a growth curve is shown in Fig. 1.9.

Determination of cell numbers

In the diagnostic laboratory, the number of bacterial cells present is determined in one of three ways:

- *Direct counting under the microscope:* This method can be used to estimate the number of bacteria present in a specimen. It does not distinguish between live and dead cells.
- *Direct plate count:* By growing dilutions of broth cultures on agar plates, one can determine the number of colony-forming units per milliliter (CFU/mL). This method provides a count of viable cells only. It is used in determining the bacterial cell count in urine cultures.
- *Density measurement:* The density (referred to as turbidity) of a bacterial broth culture can be correlated to the number of CFU/mL of the culture. This method is used to prepare a standard inoculum for antimicrobial susceptibility testing.

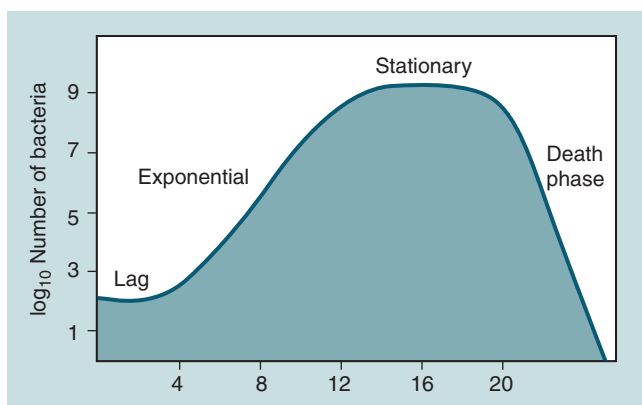


Fig. 1.9 Typical growth curve of a bacterial culture.

Bacterial biochemistry and metabolism

Metabolism

Microbial metabolism consists of the biochemical reactions bacteria use to break down organic compounds (catabolism) and the reactions they use to synthesize new molecules (anabolism) from smaller subunits. Energy for anabolism is generated during catabolism of a substrate. The occurrence of all biochemical reactions in the cell depends on the presence and activity of enzymes. Thus metabolism can be regulated in the cell either by regulation of the production of an enzyme itself (a genetic type of regulation in which production of the enzyme can be induced or suppressed by molecules present in the cell) or by regulation of the activity of the enzyme (via feedback inhibition in which the products of the enzymatic reaction or a succeeding enzymatic reaction inhibit the activity of the enzyme).

Bacteria differ widely in their ability to use various compounds as substrates and in the end products generated. Many biochemical pathways exist for catabolism in the microbial world, and the particular pathway used determines the end product and resulting pH of the medium (Fig. 1.10). Microbiologists use these metabolic differences as phenotypic markers in the identification of bacteria. Diagnostic schemes analyze each unknown microorganism for (1) utilization of various substrates as a carbon source, (2) production of specific end products from various substrates, and (3) production of an acid or alkaline pH in the test medium. Knowledge of the biochemistry and metabolism of bacteria is important in identifying microorganisms in the clinical laboratory.

Fermentation and respiration

Bacteria use biochemical pathways to catabolize carbohydrates and produce energy by two mechanisms—fermentation and aerobic respiration (commonly referred to as *oxidation*). **Fermentation** is an anaerobic process carried out by obligate, facultative, and aerotolerant anaerobes. In fermentation, the

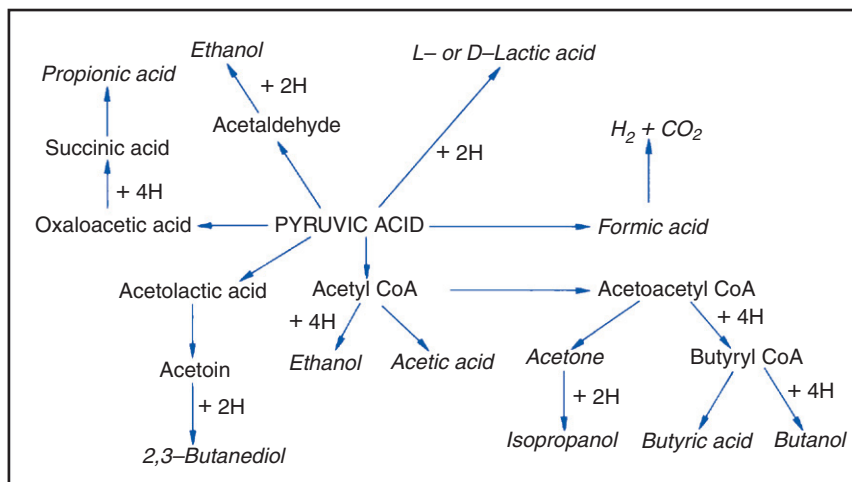


Fig. 1.10 The fate of pyruvate in major fermentation pathways of microorganisms. (From Zinsser, H., et al. [1992]. *Zinsser microbiology* [20th ed]. Norwalk, CT: Appleton & Lange.)

electron acceptor is an organic compound. Fermentation is less efficient in energy generation than respiration because the beginning substrate is not completely reduced; therefore all of the energy in the substrate is not released.

Besides allowing growth in the absence of atmospheric oxygen, fermentation is also important because it generates nicotinamide adenine dinucleotide (NAD), a molecule necessary for maintaining the Krebs cycle. When fermentation occurs, a mixture of end products (e.g., lactate, butyrate, ethanol, and acetoin) accumulates in the medium. Analysis of these end products is particularly useful for the identification of anaerobic bacteria. End-product determination is also used in the Voges-Proskauer (VP) and methyl red tests, two important tests used in the identification of members of the order Enterobacterales. The term *fermentation* is often used loosely in the diagnostic microbiology laboratory to indicate any type of utilization—fermentative or oxidative—of a carbohydrate (sugar) with the resulting production of an acid pH.

Aerobic respiration (oxidation) is an efficient energy-generating process in which molecular oxygen (O_2) is the final electron acceptor. Obligate aerobes and facultative anaerobes undergo aerobic respiration. Some anaerobes can carry out anaerobic respiration in which molecules other than molecular oxygen, such as nitrate and sulfate, act as the final electron acceptors. Anaerobic respiration is less energy yielding than aerobic respiration.

Biochemical pathways from glucose to pyruvic acid

The starting carbohydrate for bacterial fermentation or oxidation is glucose. When bacteria use other sugars as a carbon source, they first convert the sugar to glucose, which is processed by one of three pathways. These pathways are designed to generate pyruvic acid, a key three-carbon intermediate. The three major biochemical pathways bacteria use to break down glucose to pyruvic acid are (1) the Embden-Meyerhof-Parnas (EMP) glycolytic pathway (Fig. 1.11), (2) the pentose phosphate pathway (Fig. 1.12), and (3) the Entner-Doudoroff pathway (see Fig. 1.12). Pyruvate can be further catabolized either fermentatively or oxidatively. The three major metabolic pathways and their key characteristics are described in Box 1.1.

Anaerobic utilization of pyruvic acid (fermentation)

Pyruvic acid is a key metabolic intermediate. Bacteria process pyruvic acid further using various fermentation pathways. Each pathway yields different end products, which can be analyzed and used as phenotypic markers (see Fig. 1.10). Some fermentation pathways used by the microbes that inhabit the human body are as follows:

- **Alcoholic fermentation:** The major end product is ethanol. This is the pathway used by yeasts when they ferment glucose.
- **Homolactic fermentation:** The end product is almost exclusively lactic acid. Members of the genus *Streptococcus* and

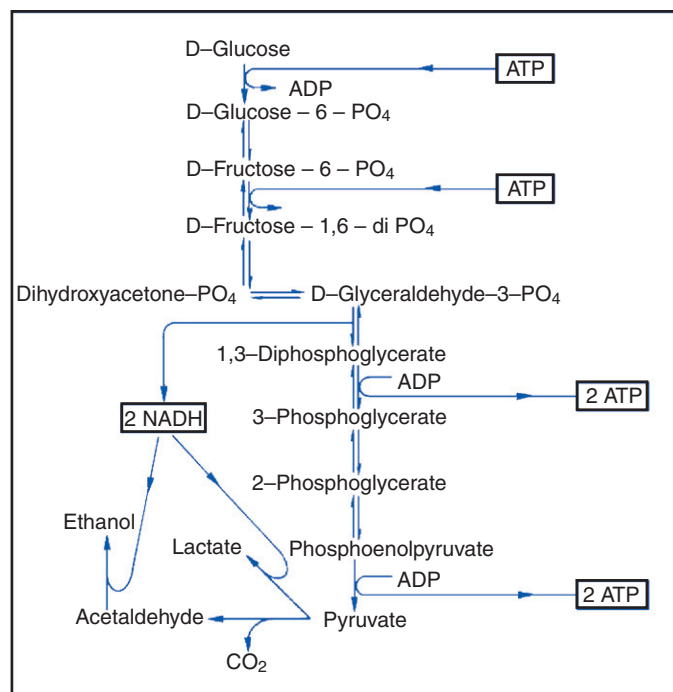


Fig. 1.11 Embden-Meyerhof-Parnas glycolytic pathway. (From Zinsser, H., et al. [1992]. *Zinsser microbiology* [20th ed]. Norwalk, CT: Appleton & Lange.)

many members of the genus *Lactobacillus* ferment pyruvate using this pathway.

- **Heterolactic fermentation:** Some lactobacilli use a mixed fermentation pathway, of which, in addition to lactic acid, the end products include carbon dioxide, alcohols, formic acid, and acetic acid.
- **Propionic acid fermentation:** Propionic acid is the major end product of fermentation carried out by *Cutibacterium acnes* (formerly *Propionibacterium acnes*) and some anaerobic non-spore-forming, gram-positive bacilli.
- **Mixed acid fermentation:** Members of the genera *Escherichia*, *Salmonella*, and *Shigella* within the order Enterobacterales use this pathway for carbohydrate fermentation and produce a number of acids as end products: lactic, acetic, succinic, and formic. The strong acid produced is the basis for the positive reaction on the methyl red test exhibited by these organisms and the acidic pH on laboratory media.
- **Butanediol fermentation:** Members of the genera *Klebsiella*, *Enterobacter*, and *Serratia* within the order Enterobacterales use this pathway for carbohydrate fermentation. The end products are acetoin (acetyl methyl carbinol) and 2,3-butanediol. Detection of acetoin is the basis for the positive VP reaction characteristic of these microorganisms. Little acid is produced by this pathway. Thus organisms that have a positive VP reaction usually have a negative methyl red test, and vice versa.
- **Butyric acid fermentation:** Certain obligate anaerobes, including many *Clostridium*, *Fusobacterium*, and *Eubacterium* species, produce butyric acid as their primary end product along with acetic acid, carbon dioxide, and hydrogen.

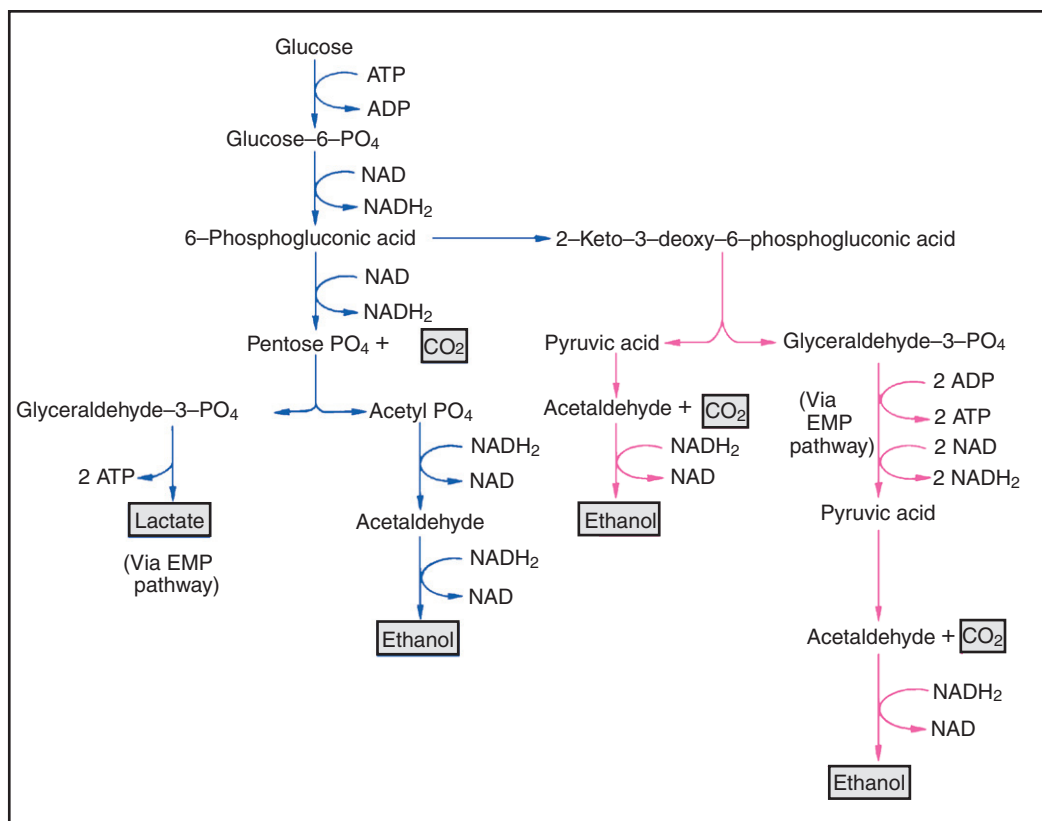


Fig. 1.12 Alternative microbial pathways to the Embden-Meyerhof-Parnas (EMP) pathway for glucose fermentation. The pentose phosphate pathway (*left*) and the Entner-Doudoroff pathway (*right*) are shown. (From Zinsser, H., et al. [1992]. *Zinsser microbiology* [20th ed]. Norwalk, CT: Appleton & Lange.)

BOX 1.1 Three major metabolic pathways found in bacteria

EMP glycolytic pathway (see Fig. 1.11)

- Major pathway in conversion of glucose to pyruvate
- Generates reducing power in the form of NADH_2
- Generates energy in the form of ATP
- Anaerobic; does not require oxygen
- Used by many bacteria, including all members of the order Enterobacterales

Pentose phosphate (phosphogluconate) pathway (see Fig. 1.12)

- Alternative to EMP pathway for carbohydrate metabolism
- Conversion of glucose to ribulose-5-phosphate, which is rearranged into other 3-, 4-, 5-, 6-, and 7-carbon sugars
- Provides pentoses for nucleotide synthesis
- Produces glyceraldehyde-3-phosphate, which can be converted to pyruvate

- Generates NADPH, which provides reducing power for biosynthetic reactions
- May be used to generate ATP (yield is less than with EMP pathway)
- Used by heterolactic fermenting bacteria, such as lactobacilli, and by *Brucella abortus*, which lacks some of the enzymes required in the EMP pathway

Entner-doudoroff pathway (see Fig. 1.12)

- Converts glucose-6-phosphate (rather than glucose) to pyruvate and glyceraldehyde phosphate, which can be funneled into other pathways
- Generates one NADPH per molecule of glucose, but uses one ATP
- Aerobic process used by *Pseudomonas*, *Alcaligenes*, *Enterococcus faecalis*, and other bacteria lacking certain glycolytic enzymes

ATP, Adenosine triphosphate; EMP, Embden-Meyerhof-Parnas; NADH_2 , nicotinamide adenine dinucleotide dehydrogenase; NADPH, nicotinamide adenine dinucleotide phosphate.

Aerobic utilization of pyruvate (oxidation)

The most important pathway for the complete oxidation of a substrate is the **Krebs cycle** or tricarboxylic acid cycle. In this cycle, the starting molecule, pyruvate, is oxidized, carbon skeletons for biosynthetic reactions are created,

guanosine-triphosphate (GTP) is generated, and electrons are donated to form NADH and FADH_2 , which ultimately enter the electron transport chain to generate energy in the form of ATP. This cycle results in the production of acid and the evolution of carbon dioxide during aerobic respiration (Fig. 1.13).

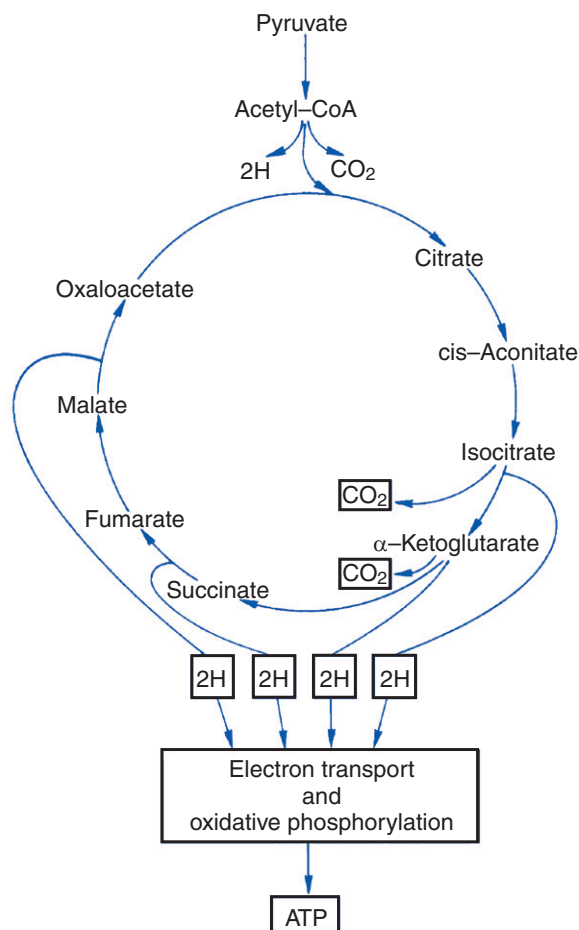


Fig. 1.13 Krebs tricarboxylic acid cycle allowing complete oxidation of a substrate. (From Zinsser, H., et al. (1992). *Zinsser microbiology* [20th ed]. Norwalk, CT: Appleton & Lange.)

Carbohydrate utilization and lactose fermentation

The ability of microorganisms to use various sugars (carbohydrates) for growth is an integral part of many identification schemes. The fermentation of the sugar is usually detected by acid production and a concomitant change of color resulting from a pH indicator present in the culture medium. Bacteria generally ferment glucose preferentially over other sugars, so glucose must be absent if the ability to ferment another sugar is being tested.

The microorganism's ability to ferment lactose is an important step in classifying members of the order Enterobacterales. These bacteria are classified as either lactose fermenters or nonlactose fermenters. Lactose is a disaccharide consisting of one molecule of glucose and one molecule of galactose linked by a galactoside bond. The utilization of lactose by a bacterium requires two steps. The first step requires the enzyme β -galactoside permease for the transport of lactose across the cell wall into the cytoplasm. The second step occurs inside the cell and requires the enzyme β -galactosidase to break the galactoside bond, releasing glucose, which then can be fermented. Thus all organisms that ferment lactose can also ferment glucose.

Microbial genetics

No discussion of microbial genetics is complete without first describing DNA and RNA. DNA was first discovered by Frederick Miescher in 1869. In the 1920s, Phoebus A. T. Levine discovered that DNA contained phosphates, five-carbon sugars (cyclic pentose), and nitrogen-containing bases. Later, Rosalind Franklin discovered the helical structure by x-ray crystallography. Most everyone is familiar with James Watson and Francis Crick, who described the three-dimensional structure of the DNA molecule in the 1950s.

Anatomy of a DNA and RNA molecule

DNA is a double-helical chain of deoxynucleotides. The helix is a double strand twisted together, which many scientists refer to as a *spiral staircase* (resembling the handrail, sides, and steps of a spiral staircase). A nucleotide is a complex combination of the following:

- A phosphate group (PO_4)
- A cyclic five-carbon pentose (the carbons in the pentose are numbered 1' to 5') sugar (deoxyribose), which makes up the "handrails and sides"
- A nitrogen-containing base, or the "steps," either a purine or a pyrimidine

A purine consists of a fused ring of nine carbon atoms and nitrogen. There are two purines in DNA: adenine (A) and guanine (G). A pyrimidine consists of a single ring of six atoms of carbon and nitrogen. There are two pyrimidines in DNA: thymine (T) and cytosine (C). A nucleotide is formed when the 5' carbon of the sugar and one of the nitrogenous bases attaches to the 1' carbon of the pentose sugar. These are the basic building blocks of DNA (Fig. 1.14).

In the chain of deoxynucleotides, bonds form between the phosphate group of one nucleotide and the 3' sugar of the next nucleotide. The base extends from the sugar. Adenine of one chain always pairs with thymine of the other chain, and cytosine of one chain pairs with guanine of the other chain. The bases are held together by hydrogen bonds. The information contained in DNA is determined by the sequence of letters along the "staircase." The sequence ACGCT represents different information than the sequence AGTCC. This would be like taking the word *stops* and using the same letters to form the word *spots* or *posts*, which have different meanings but all of the same letters.

The two complementary sugar phosphate strands run in opposite directions (antiparallel), 3' to 5' and 5' to 3', similar to one train with its engine going one way alongside a caboose of a train going the opposite direction (Fig. 1.15). The direction is based on what is found at the ends of the strands; for example, phosphate attaches to the 5' carbon of the sugar, and the OH group is attached to the 3' carbon of the sugar.

DNA is used as a template to produce the complementary messenger RNA (mRNA) strand. In RNA, the nitrogenous base thymine is replaced by uracil, another pyrimidine. In contrast with DNA, RNA is single-stranded and short, not double-stranded and long, and it contains the sugar ribose, not deoxyribose.

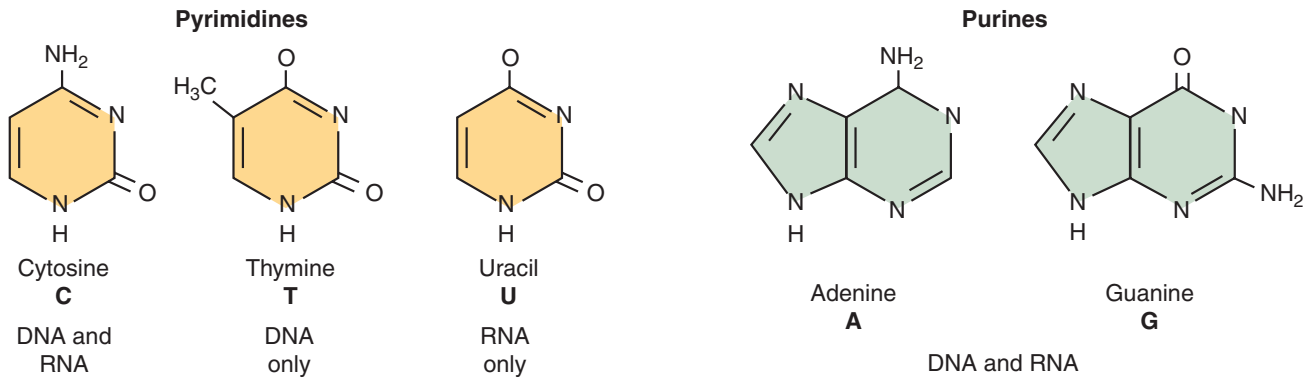


Fig. 1.14 Molecular structure of nucleic acid bases. Pyrimidines: cytosine, thymine, and uracil. Purines: adenine and guanine.

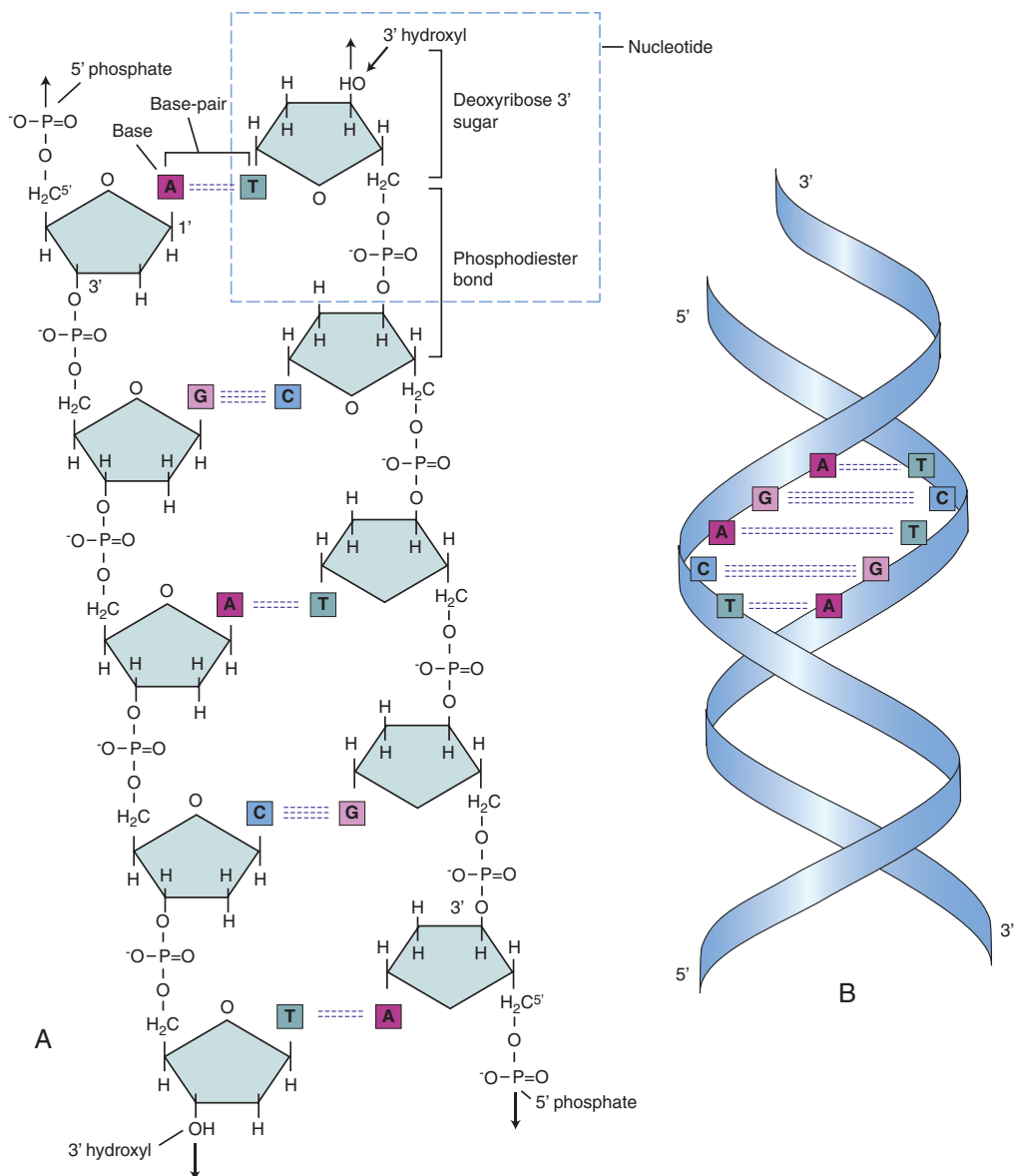


Fig. 1.15 A, Molecular structure of DNA showing nucleotide structure, a phosphodiester bond connecting nucleotides, and complementary pairing of bases (A, adenine; T, thymine; G, guanine; C, cytosine) between antiparallel nucleic acid strands. **B**, 5' and 3' antiparallel polarity and “twisted ladder” configuration of DNA.

Humans are 99.9% identical. In a human genome of 3 billion “letters,” even one tenth of 1% translates into 3 million separate lettering differences, an important characteristic useful in forensic science. Microbial genetics is increasingly important in the diagnostic microbiology laboratory. Identification tests have been developed that are based on identifying unique RNA or DNA sequences present in each bacterial species and viruses. The polymerase chain reaction technique is a means of amplifying specific DNA sequences and detecting very small numbers of bacteria present in a specimen. Genetic tests circumvent the need to culture bacteria, providing a more rapid method of identifying pathogens.

An understanding of microbial genetics is also necessary to understand the development and transfer of antimicrobial resistance by bacteria and mutations in viruses. Viruses constantly change, as in the case of the recently identified SARS-CoV-2 (severe acute respiratory syndrome–coronavirus-2), the virus that causes COVID-19 (Coronavirus Disease 2019). COVID-19 is the respiratory illness responsible for the COVID-19 pandemic that began in 2020. The occurrence of mutations can result in a change in the expected phenotypic characteristics of an organism and provides an explanation for atypical results sometimes encountered on diagnostic biochemical tests. For example, mutations in the SARS-CoV-2 virus present in a patient specimen can potentially affect test performance influenced by the sequence of the variant, the design of the test, and the prevalence of the variant in the population. To learn more about the newly identified SARS-CoV-2 virus, see [Chapter 29](#).

This section briefly reviews some of the basic terminology and concepts of microbial genetics. For a detailed discussion of DNA and molecular diagnostics, see [Chapter 11](#).

Terminology

The genotype of a cell is the genetic potential of the DNA of an organism. It includes all of the characteristics that are coded for in the DNA of a cell and that have the potential to be expressed. Some genes are silent genes, expressed only under certain conditions. Genes that are always expressed are *constitutive*. Genes that are expressed only under certain conditions are *inducible*. The phenotype of a cell consists of observed characteristics expressed by the genome. The aim of a cell is to produce proteins that are needed for cellular structure and function and to transmit that information for accomplishing this to the next generation of cells. Information for protein synthesis is encoded in the DNA and transmitted in the chromosome to each generation. The general flow of information in a cell is from DNA (which contains the genetic information) to mRNA, which acts as a blueprint for protein construction.

Replication is the duplication of chromosomal DNA for insertion into a daughter cell. **Transcription** is the synthesis of mRNA by the enzyme RNA polymerase using one strand of DNA as a template. **Translation** is the synthesis of a specific protein from the mRNA code. The term **protein expression** also refers to the synthesis of a protein. Proteins are polypeptides composed of amino acids. The number and sequence of amino acids in a polypeptide and the character

of that particular protein are determined by the sequence of codons in the mRNA molecule. A **codon** is a group of three nucleotides in an mRNA molecule that signifies a specific amino acid. During translation, ribosomes containing rRNA sequentially add amino acids to the growing polypeptide chain. These amino acids are brought to the ribosome by transfer RNA (tRNA) molecules that “translate” the codons. The tRNA molecules temporarily attach to mRNA using their complementary anticodon regions. An **anticodon** is the triplet of bases on the tRNA that bind the triplet of bases (codon) on the mRNA. It identifies which amino acid will be in a specific location in the protein.

Genetic elements and alterations

Bacterial genome

The bacterial chromosome, also called the *genome*, consists of a single, closed, circular piece of dsDNA that is supercoiled to fit inside the cell. It contains all of the information needed for cell growth and replication. Genes are specific DNA sequences that code for the amino acid sequence in one protein (e.g., one gene equals one polypeptide), but this may be sliced or combined with other polypeptides to form more than one protein. In front of each gene on the DNA strand is an untranscribed area containing a promoter region, which the RNA polymerase recognizes and binds to for transcription initiation. This area may also contain regulatory regions to which molecules may attach and cause either a decrease or an increase in transcription.

Extrachromosomal DNA elements

In addition to the genetic information encoded in the bacterial chromosome, many bacteria contain additional information on small circular pieces of *extrachromosomal* dsDNA called **plasmids**. They are *not* essential for bacterial growth, so they can be gained or lost. Genes that code for antimicrobial resistance (and sometimes toxins or other virulence factors) are often located on plasmids. Antimicrobial therapy selects for bacterial strains containing plasmids encoding drug-resistance genes; this is one reason antimicrobial agents should not be overprescribed. The number of plasmids present in a bacterial cell may range from one (low copy number) to hundreds (high copy number). Plasmids are located in the cytoplasm of the cell and are self-replicating and passed to daughter cells, similar to chromosomal DNA. They also may sometimes be passed from one bacterial cell to another through **conjugation** (horizontal transfer of genetic material by cell-to-cell contact). This is one way drug resistance is acquired.

Mobile genetic elements

Some fragments of DNA are mobile and can jump from one place in the chromosome to another place. These are sometimes referred to as *jumping genes*. The simplest mobile piece of DNA is an insertion sequence (IS) element. It is approximately 1000 base pairs long with inverted repeats on each end. Each IS element codes for only one gene, a transposase enzyme that allows the IS element to pop into and out of

DNA. Bacterial genomes contain many IS elements. The main effect of IS elements in bacteria is that when an IS element inserts itself into the middle of a gene, it disrupts and inactivates the gene. This action can result in loss of an observable characteristic, such as the ability to ferment a particular sugar. Transposons are related mobile elements that contain additional genes. Transposons often carry drug-resistance genes and are usually located in plasmids.

Mutations

Mutations are changes that occur in the DNA code and often result in a change in the coded protein or in the prevention of its synthesis. Some mutations are silent, in which a change in the DNA sequence does not result in the substitution of a different amino acid in the resulting protein. This is caused by redundancy in protein synthesis; more than one codon codes for the same amino acid. A mutation may be the result of a change in one nucleotide base (a point mutation) that leads to a change in a single amino acid within a protein (missense mutation) or may be the result of insertions or deletions in the genome that lead to disruption of the gene or a frameshift mutation, or both. A gene sequence must be read in the right “frame,” or series of three codons, for the correct protein to be produced. This is because every set of three bases in mRNA (a codon) specifies a particular amino acid, and when the reading frame is shifted, the codons are also shifted. Incomplete, inactive proteins are often the result of frameshift mutations. Some codons do not code for an amino acid; they terminate translation and are called *stop codons*. If a point mutation produces a stop codon, this is referred to as a *nonsense mutation*. Spontaneous mutations occur in bacteria at a rate of about 1 in 10^9 cells. Mutations also occur as the result of error during DNA replication at a rate of about 1 in 10^7 cells. Exposure to certain chemical and physical agents can greatly increase the mutation rate.

Genetic recombination

Genetic recombination is a method by which genes are transferred or exchanged between homologous (similar) regions on two DNA molecules, forming new combinations of genes on a chromosome. This provides a way for organisms to obtain new combinations of biochemical pathways and adapt to changes in their environment.

Mechanisms of gene transfer

Genetic material may be transferred from one bacterium to another in three ways:

- Transformation
- Transduction
- Conjugation

Transformation

Transformation is the uptake and incorporation of free or naked DNA into a bacterial cell (Fig. 1.16A). Once the DNA has been taken up, it can be incorporated into the bacterial genome by recombination. If the DNA is a circular plasmid

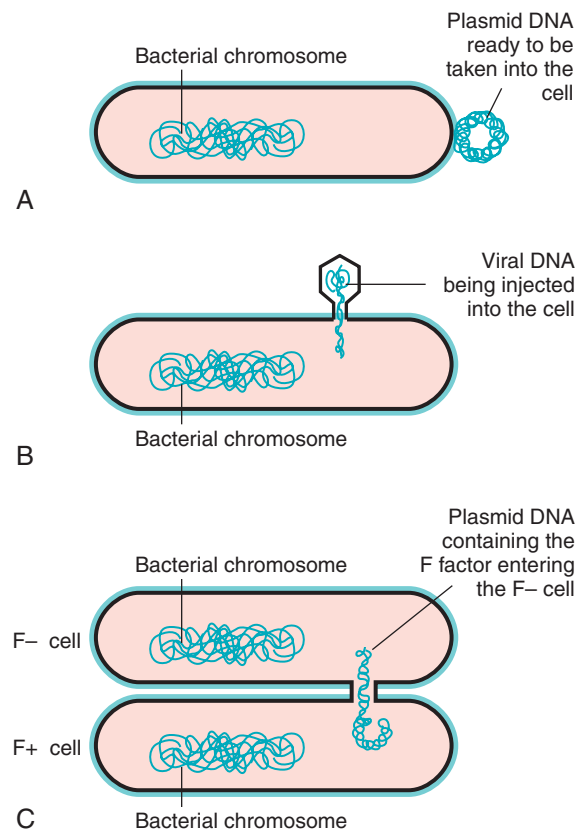


Fig. 1.16 Methods of gene transfer into bacterial cells. **A**, Bacterial transformation. Free or “naked” DNA is taken up by a competent bacterial cell. After uptake, the DNA may take one of three courses: (1) it is integrated into existing bacterial genetic material; (2) it is degraded; or (3) if it is a compatible plasmid, it may replicate in the cytoplasm. **B**, Bacterial transduction. A phage injects DNA into the bacterial cell. The phage tail binds to a receptor of the bacterial cell wall and injects the DNA into the bacterium. One of two courses may then occur. In the lytic cycle, replication of the bacterial chromosome is disrupted; phage components are formed and assembled into phage particles. The bacterial cell is lysed, releasing a mature phage. In the lysogenic cycle, the phage DNA is incorporated into the bacterial genetic material, and genes encoded by the phage DNA can be expressed. At a later time, the phage may be “induced,” and a lytic cycle then ensues. **C**, Bacterial conjugation. An F+ cell connects with an F- cell via sex pili. DNA is then transferred from the F+ cell to the F- cell.

and the recipient cell is compatible, the plasmid can replicate in the cytoplasm and be transferred to daughter cells during cell division. Cells that can take up naked DNA are referred to as being **competent**. Transformation is not an efficient process and has been reported to occur naturally in only a few bacterial species, such as *S. pneumoniae*, *N. gonorrhoeae*, *Helicobacter pylori*, and *H. influenzae*. Bacteria in biofilms have an increased rate of transformation compared with bacteria in the planktonic state. Bacteria can be made competent in the laboratory, and transformation is the main method used to introduce genetically manipulated plasmids into bacteria, such as *E. coli*, during cloning procedures.

Transduction

Transduction is the transfer of bacterial genes by a bacteriophage from one cell to another (see Fig. 1.16B). A bacteriophage consists of genetic material (DNA or RNA) surrounded

by a protein coat. When a phage infects a bacterial cell, it injects its genome into the bacterial cell, leaving the protein coat outside. The phage can then take a lytic pathway in which the bacteriophage DNA directs the bacterial cell to synthesize the phage genome and proteins and package it into new phage particles. The bacterial cell eventually lyses (lytic phase), releasing new phage particles that can infect other bacterial cells.

In some instances, the phage DNA instead becomes incorporated into the bacterial genome, where it is replicated along with the bacterial chromosomal DNA when the cell divides. This state is known as **lysogeny**, and the phage is referred to as being **temperate**. During lysogeny, genes present in the phage DNA may be expressed by the bacterial cell. An example of this in clinical microbiology is *C. diphtheriae*. Strains of *C. diphtheriae* that are lysogenized with a temperate phage carrying the gene for diphtheria toxin cause disease. Strains lacking the phage DNA do not produce the toxin and do not cause disease.

Under certain conditions, a temperate phage can be induced, the phage DNA is excised from the bacterial genome, and a lytic state occurs. During this process, adjacent bacterial genes may be excised with the phage DNA and packaged into the new phages. The bacterial genes may be transferred when the phage infects a new bacterium. In the field of biotechnology, phages are often used to insert cloned genes into bacteria for analysis.

Conjugation

Conjugation is the transfer of genetic material from a donor bacterial strain to a recipient strain (see Fig. 1.16C). Close contact is required between the two cells. In *E. coli*, the donor strain (F+) possesses fertility factor (F factor) on a plasmid that carries the genes for conjugative transfer. The donor strain produces a hollow surface appendage called a *sex* or *conjugation pilus*, which binds to the recipient F− cell and brings the two cells in close contact. Transfer of DNA then occurs. Both plasmids and chromosomal genes can be transferred by this method. When the F factor is integrated into the bacterial chromosome rather than a plasmid, there is a higher frequency of transfer of adjacent bacterial chromosomal genes. These strains are known as *high-frequency recombination strains*.

Restriction enzymes

Bacteria have evolved a system to restrict the incorporation of foreign DNA into their genomes. **Restriction enzymes** are produced that cut (restrict) incoming, foreign DNA at specific DNA sequences. The bacteria methylate their own DNA at these same sequences, so the restriction enzymes do not cut the DNA in their own cell. Many restriction enzymes with various recognition sequences have been isolated from various microorganisms. The first three letters in the restriction endonuclease name indicate the bacterial source of the enzyme. For instance, the enzyme *EcoRI* was isolated from *E. coli*, and the enzyme *Hind III* was isolated from *H. influenzae* type d. These enzymes are used in the field of biotechnology to create sites for insertion of new genes.

POINTS TO REMEMBER

- Many microorganisms inhabit the environment, and most are nonpathogenic.
- Prokaryotes, such as bacteria, do not have membrane-enclosed nuclei and organelles.
- Eukaryotes differ from prokaryotes in that they have membrane-enclosed nuclei and organelles.
- Viruses cannot be seen under an ordinary light microscope, although their cytopathic effects on cell cultures are visible. They are obligate parasites, and antimicrobial agents are ineffective for treatment of viral infections. Viruses have DNA or RNA, but rarely both, in contrast with prokaryotes and eukaryotes.
- In the diagnostic microbiology laboratory, the first step in identifying bacteria is by the Gram stain reaction. Whether an organism is gram-positive (blue or purple) or gram-negative (pink or red) depends on its cell wall structure. A thick peptidoglycan layer is present in gram-positive bacteria, while an outer membrane and thin peptidoglycan layer are found in gram-negative bacteria.
- Bacterial spores are formed because of harsh environments. They are a means of survival, not reproduction.
- The LPS contained in the outer membrane of gram-negative bacteria consists of three regions: an antigenic O-specific polysaccharide, a core polysaccharide, and an inner lipid A (also called *endotoxin*). The lipid A moiety is responsible for producing fever and shock conditions in patients infected with gram-negative bacteria.
- Based on atmospheric requirements, bacteria are classified as obligate aerobes, obligate anaerobes, facultative anaerobes, aerotolerant anaerobes, or microaerophiles.
- Bacteria utilize two biochemical pathways, fermentation and oxidation (aerobic respiration), to catabolize carbohydrates to produce energy.
- The three mechanisms by which genetic material may be transferred from one bacterium to another are transformation, transduction, and conjugation.

LEARNING ASSESSMENT QUESTIONS

1. Explain why the laboratory scientist in the Case in Point should repeat the Gram stain procedure on the exudate.
2. What might have occurred to make the Gram stain results invalid?
3. Differentiate the role of pili from the role of flagella.
4. What is the role of the bacterial capsule in the pathogenesis of infectious diseases?
5. Why is lipopolysaccharide (LPS) a significant outer-membrane structure in gram-negative bacteria?
6. A bacterium that grows only on plates incubated in the absence of oxygen would be categorized as a(n):
 - a. Aerotolerant anaerobe.
 - b. Facultative anaerobe.
 - c. Obligate anaerobe.
 - d. Obligate aerobe.
7. Fimbriae present on the outer surface of bacteria are used for:
 - a. Adherence to surfaces.
 - b. Antimicrobial resistance.
 - c. Sexual reproduction.
 - d. Bacterial motility.

8. All of the following are characteristic of fermentation *except*:
 - a. It begins with the breakdown of pyruvic acid.
 - b. It follows glycolysis and produces reduced nicotinamide adenine dinucleotide (NADH).
 - c. It produces acids, alcohols, and gases.
 - d. It can occur in the presence of oxygen.
9. Why are older bacterial cells more easily decolorized than cells from younger colonies?
10. Why are spore-forming organisms more resistant to environmental stress than non-spore-forming species?
11. Explain the three ways in which genetic material can be transferred from one bacterium to another.
12. For the following DNA, write the complementary sequence. Include labeling the 3' and 5' end. 3' TTACGGACAAC 5':
_____.
13. In RNA, thymine is replaced by _____.
14. In bacteriophage, how does lysogeny differ from the lytic cycle?
15. Nutrient and trypticase soy broths are culture media that are used primarily to satisfy the growth requirements of bacteria and support the growth of most nonfastidious organisms. This type of media is classified as:
 - a. Nutritive.
 - b. Selective.
 - c. Differential.
 - d. Enriched.
16. A patient was seen in the emergency department with fever and symptoms of disseminated intravascular coagulation, sepsis, and shock. Blood cultures taken from the patient grew gram-negative bacteria. Which of the following cellular structures might be responsible for this condition?
 - a. Pili
 - b. Capsule
 - c. Endotoxin
 - d. Spore
17. Which of the following best describes an organism that cannot use oxygen but will not be killed by oxygen if exposed to it?
 - a. Aerotolerant anaerobe
 - b. Obligate anaerobe
 - c. Facultative anaerobe
 - d. Obligate aerobe
18. An encapsulated organism is considered to be virulent because it can mediate which of the following?
 - a. Form spore
 - b. Inhibit phagocytosis
 - c. Increase mutation rate
 - d. Enhance motility

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Host-parasite interaction

Donald C. Lehman

CHAPTER OUTLINE

Origin of microbial biota, 26

- Characteristics of indigenous microbial biota, 26
- Factors that determine the composition of the usual microbial biota, 27

Composition of microbial biota at different body sites, 27

- Normal microbiota of the skin, 27
- Normal microbiota of the oral cavity, 28
- Normal microbiota of the respiratory tract, 28
- Normal microbiota of the gastrointestinal tract, 29
- Normal microbiota of the genitourinary tract, 29

Role of the microbial biota in the pathogenesis of infectious disease, 30

Role of the microbial biota in the host defense against infectious disease, 30

Microbial factors contributing to pathogenesis and virulence, 31

- Pathogenesis, 31
- Routes of transmission, 32
- Virulence, 35
- Ability to resist phagocytosis, 35
- Structures that promote adhesion to host cells and tissues, 35
- Ability to survive intracellularly and proliferate, 37
- Ability to produce extracellular toxins and enzymes, 37

Host resistance factors, 39

- Physical barriers, 39
- Cleansing mechanisms, 39
- Antimicrobial substances, 40
- Phagocytosis, 41
- Inflammation, 42
- Immune responses, 42

Mechanisms by which microbes may overcome host adaptive defenses, 47

Bibliography, 49

OBJECTIVES

After reading and studying this chapter, you should be able to:

1. Define the following terms: *parasitism*, *indigenous biota*, *commensal*, *symbiont*, *opportunist*, *transient biota*, *carrier*, *true pathogen*, *opportunistic pathogen*, *virulence*, and *zoonosis*.
2. Explain how the following factors determine the composition of the microbial biota at various body sites:
 - Amounts and types of nutrients available in the environment
 - pH
 - Oxidation-reduction potential
 - Resistance to antibacterial substances
3. List the predominant microbiota of various body sites in a healthy individual.
4. Evaluate the role of the indigenous microbiota in the pathogenesis of infectious disease.
5. Evaluate the role of the indigenous biota in host defense against infectious diseases.
6. Differentiate the mechanisms of infections caused by true pathogens from infections caused by opportunistic pathogens.
7. Name the routes of transmission that microorganisms use to initiate infection in a host, and give examples of each.
8. Discuss the conditions that must be present or events that must occur for a microorganism to cause disease.
9. Describe the characteristics of infectious agents that enable them to cause disease in the host.
10. Describe the innate and adaptive factors and mechanisms by which the human host is protected from microbial invasion.
11. Discuss the sequence of events in the phagocytosis and killing of an infectious agent.
12. Correlate a specific immune deficiency with the outcome to the host in preventing infectious diseases.

KEY TERMS

Adaptive immunity
Adhesin

Anamnestic immune response
Antibody

Antigen	Innate immunity
Bacteriocin	Interferon
Carrier	Lactoferrin
Carrier state	Leukocidin
Cell-mediated immune response	Lymphocyte
Chemotactic agent	Lymphokine
Chemotaxis	Lysozyme
Colonization	Mutualism
Commensalism	Opportunists
Complement system	Opsonin
Diapedesis	Opsonization
Dissemination	Parasite
Endotoxin	Parasitism
Exotoxin	Pathogen
Fimbriae	Pathogenicity
Fomites	Pattern recognition receptor
Humoral immune response	Phagocyte
Iatrogenic infection	Phagocytosis
Immune response	Pili
Immune system	Receptor
Immunogen	Resident microbial biota
Immunoglobulin A (IgA)	Respiratory burst
Immunoglobulin D (IgD)	Symbiosis
Immunoglobulin E (IgE)	Toll-like receptor
Immunoglobulin G (IgG)	Transient microbial biota
Immunoglobulin M (IgM)	Virulence
Immunoglobulins	Zoonoses
Indigenous microbiota	

Case in point

A 71-year-old male patient was treated for right lower extremity cellulitis with a 10-day course of the antibiotic cephalexin. A few days after completing the antibiotic course, he started having loose, watery diarrhea. The patient described having many episodes of diarrhea per day; after 3 days of diarrhea, he came to the emergency department. He also said that he had developed right quadrant abdominal pain over the past 24 hours. While in the emergency department, the patient had five episodes of loose, watery diarrhea. A bacterial culture of the stool was negative for *Salmonella*, *Shigella*, *Campylobacter*, *Yersinia*, and *Vibrio* species, but a polymerase chain reaction assay for the *Clostridioides difficile* toxin B gene was positive.

Issues to consider:

After reading the patient's case history, consider:

- Factors that predisposed this patient to his current condition
- Clues that indicate the source of the infection
- Significance of the microbiota in protecting the host against pathogenic organisms
- Role of the host's innate and acquired immunity in protecting the host from infection
- Significance of microbial virulence factors in promoting infection

The outcome from the interactions between host and **pathogen**, a disease-causing agent, is influenced by numerous factors. The status of the host's immune system and ability of the host to defend itself from microbial and viral invasion, combined

with microbial and viral factors inherent to the invading pathogen, often determine whether disease occurs. To appreciate and understand the concepts involved in the pathogenesis of infectious diseases, knowledge and understanding of the host-pathogen relationship is important.

It is beyond the scope of this book to provide a detailed discussion of the complexities of the immune response. This chapter provides an overview by describing the interactions between the host and infectious agents in the pathogenesis of disease. The first part of the chapter describes the origin of the indigenous (resident) microbial biota (microbiota) and the composition at different body sites. It presents the role of the microbial biota at each body site in the host immune defense and as a source of opportunistic infections. Factors that determine the composition of the microbiota at different body sites are described. The second part of the chapter discusses the virulence factors that contribute to the invasiveness of organisms, protective mechanisms the host employs, and how microbes can evade the host's defenses. Lastly, this chapter describes factors that can make the host more susceptible to infections and how microbes are transmitted.

Origin of microbial biota

The fetus is in a sterile environment. During delivery and the first few days of life, the newborn is introduced to the many and varied microorganisms present in the environment. Many organisms can find an area on or in the infant into which they can adapt. Microorganisms that find their niche colonize various anatomic sites and become the predominant organisms. **Colonization** is growth of microbiota in or on a body site without the production of damage or notable symptoms. Other microorganisms are transient or fail to establish themselves at all. As the infant grows, the microbial biota eventually resemble the microbiota seen in older individuals.

Once established onto or into a particular body site of the host, microorganisms develop a particular relationship, or **symbiosis**, with that host. *Symbiosis* is defined as the association of two organisms of different species living together. The organisms are called *symbionts*. The host-microbe relationship, depending on the circumstance, may be one of **commensalism**, **mutualism**, or **parasitism**. Symbiosis as a biological relationship in which both (host and organism) benefit from one another may be described as *mutualism*. Lactobacilli in the urogenital tract of women offer a mutual association: the lactobacilli provide the host protection by preventing colonization of pathogenic species at that site while they derive nutrients from the host. In the relationship in which the organism benefits but there is no beneficial or harmful effect on the host, the association is called *commensalism*. *Klebsiella pneumoniae* is a commensal species in the gastrointestinal tract of humans. In parasitism, one species (parasite) benefits at the expense of the other (host). The parasite *Entamoeba histolytica* is a pathogenic intestinal amoeba that derives nutrients from the host at the expense of the host, causing intestinal ulcers and amebic dysentery.

Characteristics of indigenous microbial biota

Microorganisms that are commonly found on or in body sites of healthy persons are called *normal* or **indigenous microbiota**. The different body sites may have the same or different microbiota,

depending on conditions. Local conditions select for organisms that are suited for growth in a particular area. For example, the environment found on the dry skin surface is different from the environment found on the moist surfaces in the oral cavity; therefore the microbiota are different at the two sites.

Microorganisms that colonize an area for months or years represent **resident microbiota**, whereas microorganisms that are present at a site temporarily, for a shorter period, represent **transient microbiota**. Transient microbiota come to “visit” but do not stay. These microorganisms are eliminated either by the host-inherent immune defenses or by competition with the resident biota. Some pathogenic organisms may establish themselves in a host without manifesting symptoms. However, these hosts, called carriers, can transmit the infectious agent. The condition of these hosts is called the carrier state. The carrier state may be acute (short-lived) or chronic (lasting for months, years, or permanently). An example of a chronic carrier state is found in post-*Salmonella* Typhi infection. This organism can establish itself in the bile duct and can be excreted in the stool over years. In contrast, *Neisseria meningitidis* can be found in the nasopharynx of asymptomatic individuals during an outbreak of meningitis. After a few days or weeks at most, these individuals may no longer harbor the organism, in which case the carrier state would be termed *acute*. The most transient of carrier states is the inoculation of a person’s hands or fingers with an organism, for example, *Staphylococcus aureus* that has colonized the person’s anterior nares and is transmitted to the hands, that is carried only until the hands are washed.

The organisms colonizing different body sites play a significant role in providing host resistance to infections. The efficiency of the microbial biota in providing protection to the human host is indicated by the relatively small number of infections caused by these organisms in immunocompetent individuals. Nevertheless, these organisms may cause significant, often serious, infections or may exacerbate existing infections in individuals who are immunocompromised. Knowing the benefit of the normal microbiota, individuals can ingest probiotics, a suspension of live bacteria that normally colonize the gastrointestinal tract, to reestablish the microbiota. This is useful in some patients who have taken oral broad-spectrum antimicrobial agents that have reduced the number of resident bacteria in the intestines.

Factors that determine the composition of the usual microbial biota

Which microorganisms are present at a particular body site is influenced by nutritional and environmental factors, such as the amount and types of nutrients available at the site. For example, more organisms inhabit moist areas than dry areas. Moist skin areas are dominated by diphtheroids, nonpathogenic corynebacteria. Although lipids and fatty acids are bactericidal to most bacteria, *Propionibacterium* spp. colonize the ducts of hair follicles because these bacteria can break down the skin lipids to fatty acids. The affinity of microorganisms for a specific site depends on the ability of the organisms to resist the antibacterial effects of substances such as fatty acids, bile, or lysozyme. The composition of the microbial biota is also affected by pH. For example, the female genital tract microbiota depends on the pH of that environment, which in women

of childbearing age is approximately 4.0 to 5.0. Many bacteria do not survive at this extreme pH range. Another example is the fecal biota found in infants who are breast-fed, which differs from the fecal biota in infants fed with cow’s milk. Human milk has a high lactose concentration and maintains a pH of 5.0 to 5.5, an environment supportive of *Bifidobacterium* spp. Cow’s milk has a greater buffering capacity and is less acidic. Infants fed with cow’s milk do not have the high colonization rate by *Bifidobacterium* spp. found in breast-fed infants but have instead a colon microbiota similar to that seen in older children and adults (see [Box 2.5](#)). In areas of the body that have a low oxidation-reduction potential, the environment supports only organisms capable of fermentation, such as is seen in the gingival crevices colonized with *Bacteroides* and *Fusobacterium*.

The previously described environmental conditions may change with age, nutritional status, disease states, and drug or antimicrobial therapy use. These changes can predispose an individual to infection by the indigenous biota, a type of infection referred to as an *opportunistic infection*. For example, two groups at increased risk for gram-negative bacillus pneumonia are diabetics and alcoholics. Also, antimicrobial agents can reduce a particular population of resident bacteria, allowing the proliferation of others. An increase in age brings with it a decrease in the effectiveness of the immune response. As a result, the incidence of infection caused by opportunistic organisms increases.

Case check 2.1

The patient in the Case in Point developed diarrhea following a course of antimicrobial therapy. As described in the text, antimicrobial therapy can alter the usual biota of nearly any body site. In this case, antimicrobial therapy altered the normal bacterial biota of the gastrointestinal tract and allowed *C. difficile* to cause an infection. In this case *C. difficile* may have been a component of this patient’s bowel biota, or, more likely, the patient acquired *C. difficile* from the environment.

Composition of microbial biota at different body sites

Human microbiome studies using molecular sequencing strategies to determine which organisms reside in or on the human body have shown that the human host is colonized by many different species of microorganisms. For example, in the oral cavity alone, approximately 500 different species have been characterized. The effectiveness of the various host defenses is evidenced by the relatively low incidence of infection in immunocompetent individuals by members of the usual or indigenous microbiota. However, infections caused by members of the microbial biota are frequently encountered among immunocompromised patients. The clinical microbiologist must be able to recognize and identify the types of microorganisms found at the various body sites.

Normal microbiota of the skin

Normal skin has numerous mechanisms to prevent infection and protect the underlying tissue from invasion by potential

pathogens. These mechanisms include a physical barrier separating microorganisms from the tissues, presence of fatty acids that inhibit many microorganisms, excretion of lysozyme by sweat glands, and desquamation of the epithelium. The skin contains a wide variety of resident microorganisms, most of which are found on the most superficial layers of skin cells and the upper parts of hair follicles. Scrubbing and washing may reduce the number of bacteria present on the skin by about 90% but do not eliminate completely the organisms present, and their numbers return to normal within a few hours.

The three types of sweat glands in humans are *eccrine*, *apocrine*, and *apoecrine*. Eccrine glands are distributed abundantly in the skin and secrete mainly water and electrolytes. Apocrine glands, found mostly in the armpits and anogenital regions, produce viscous secretions. Sebaceous glands are connected to hair follicles and secrete sebum. The composition of the microbiota on the skin depends on the activity of the sebaceous or sweat glands. Organisms concentrate the most in moist areas, such as the armpit, groin, and perineum. The apocrine glands in these areas secrete substances metabolized by the skin bacteria, releasing odorous amines—the source of “body odor.” Aerobic diphtheroids are usually found in moist areas such as the axillae and between the toes. *Staphylococcus epidermidis*, *Cutibacterium* (*Propionibacterium*) *acnes*, and *Propionibacterium* spp. reside in hair follicles and metabolize products secreted by sebaceous glands. They are resistant to skin lipids and fatty acids as well as to superficial antiseptic agents commonly used to cleanse the skin.

The presence of skin bacteria benefits the host by inhibiting the growth of more pathogenic bacterial species by competing for nutrients. In addition, *S. epidermidis* and *C. acnes* secrete bacteriocins and induce the production of antimicrobial peptides by host cells, such as keratinocytes, sebocytes, and immune cells. **Bacteriocins** are peptides produced by bacteria to inhibit the growth of similar or closely related bacteria.

Microorganisms such as *C. acnes* colonize the deep sebaceous glands. Superficial antisepsis of the skin does not eliminate this organism, which may be found as a contaminant in culture specimens obtained by invasive procedures (e.g., blood, cerebrospinal fluid) because of contamination of the needle. [Box 2.1](#) lists the microorganisms most commonly found on the skin. Other organisms have been isolated from the skin but are found only occasionally or rarely and are not listed.

BOX 2.1 Microorganisms found on the skin

Common residents

Candida spp.
Cutibacterium acnes
Micrococcus spp.
Staphylococcus spp.
Propionibacterium spp.
 Diphtheroids (*Corynebacterium* spp.)

Less common or transients

Streptococcus spp.
Acinetobacter spp.
 Gram-negative rods (fermenters and nonfermenters)
Moraxella spp.

Normal microbiota of the oral cavity

The mouth contains large numbers of bacteria, with the genus *Streptococcus* predominating. Many organisms bind to the buccal mucosa and tooth surface. Unlike skin, teeth do not have a shedding epithelium. Bacteria on teeth and along the gums form a multispecies biofilm (plaque), allowing long-term colonization. Without proper oral hygiene, this can lead to dental caries and periodontitis. The bacterial plaque that develops on teeth may contain 10^{11} streptococci per gram. Plaque also results in a low oxidation-reduction potential at the tooth surface; this supports the growth of strict anaerobes, particularly in crevices and in the areas between the teeth. [Box 2.2](#) provides a partial list of microorganisms found in the oral cavity.

Normal microbiota of the respiratory tract

The respiratory tract, commonly divided into the upper and lower respiratory tract, is responsible for the delivery of air from outside of the body to the pulmonary tissues responsible for exchange of oxygen and carbon dioxide. The upper respiratory tract is composed of the mouth, nasopharynx, oropharynx, and larynx; the lower respiratory tract is composed of the trachea, bronchi, and pulmonary parenchyma. The trachea, bronchi, and lungs are protected by the action of ciliary epithelial cells and by the movement of mucus along the trachea. The tissues of these structures are normally sterile because of this protective action.

The mouth, nasopharynx, and oropharynx are colonized predominantly with viridans streptococci such as *Streptococcus mitis* group, *S. mutans* group, *S. anginosus* group, and *S. salivarius* group. *Moraxella catarrhalis*, *Neisseria* spp., and diphtheroids also colonize the upper respiratory tract. Obligate anaerobes reside in the gingival crevices, where the anaerobic environment supports these organisms. The organisms found in the mouth, nasopharynx, oropharynx, and nose, although similar, show some differences. [Box 2.3](#) lists common microorganisms encountered in the nose and

BOX 2.2 Microorganisms found in the oral cavity

Common residents

Staphylococcus epidermidis
Streptococcus anginosus group
Streptococcus mitis group
Streptococcus salivarius group
Streptococcus mutans group
Peptostreptococcus spp.
Actinomyces israelii
Bacteroides spp.
Prevotella/*Porphyromonas*
Treponema denticola
Treponema refringens
Veillonella spp.

Less common or transients

Candida albicans
Enterococcus spp.
Eikenella corrodens
Fusobacterium nucleatum
Staphylococcus aureus

BOX 2.3 Microorganisms found in the nose and nasopharynx**Common residents**

Diphtheroids (*Corynebacterium* spp.)
Haemophilus parainfluenzae
Staphylococcus aureus
Staphylococcus epidermidis
Streptococcus spp.

Less common or transients

Haemophilus influenzae
Moraxella spp.
Neisseria meningitidis
Streptococcus pneumoniae

nasopharynx. Attachment to host cells by streptococci and staphylococci is initially caused by hydrophobic or electrostatic interactions via bacterial surface polymers, such as pili and teichoic acids. Pili are found in the pathogenic *S. pyogenes*, *S. agalactiae*, and *S. pneumoniae* but are rare among the oral streptococci.

Opportunistic pathogens such as *S. aureus*, found in approximately 30% of healthy individuals, colonize the anterior nares. The population of the nasopharynx mirrors that of the nose, although the environment is different enough from the environment of the nose to select for several additional organisms. *Haemophilus influenzae*, *S. pneumoniae*, and *N. meningitidis*, all potential pathogens, can also be found occasionally in the nasopharynx of healthy individuals. Patients hospitalized for several days might become colonized in the upper respiratory tract by gram-negative bacteria, particularly members of the order Enterobacterales. The normal biota of the oropharynx is listed in [Box 2.4](#).

Normal microbiota of the gastrointestinal tract

The gastrointestinal tract comprises the esophagus, stomach, small intestine, and colon. The gastrointestinal tract is equipped with numerous defenses and effective antimicrobial factors. Because intestinal pathogens are usually acquired by ingestion of organisms contained in contaminated food or drink, host defenses against infections are present throughout the intestinal tract. Despite the presence of antimicrobial factors, the intestinal tract is thought to be colonized by over 35,000 bacterial species. The relation between the gut microbiota and human health is being increasingly recognized.

The microorganism population is lowest in the esophagus, about 10 microbes per gram of content. Some microorganisms colonize the esophagus, and others are present in ingested food as transient biota. The stomach contains gastric juices, acids (pH 2), and enzymes that help protect the stomach from microbial attack. Many microorganisms are susceptible to the acid pH of the stomach and are destroyed, except for the spore-forming bacterial species in their spore phase and the cysts of parasites. Even with the hostile environment of the stomach, some bacteria belonging to the genera *Streptococcus*, *Enterococcus*, and *Prevotella* as well as the opportunistic pathogen *Helicobacter pylori* can inhabit the stomach. These organisms associate themselves with the stomach lining,

BOX 2.4 Microorganisms found in the oropharynx**Common residents**

α -Hemolytic and nonhemolytic streptococci
Diphtheroids (*Corynebacterium* spp.)
Staphylococcus aureus
Staphylococcus epidermidis
Streptococcus pneumoniae
Streptococcus mutans group
Streptococcus mitis group
Streptococcus anginosus group
Streptococcus salivarius group
Haemophilus parainfluenzae
Moraxella catarrhalis
Bacteroides spp.
Prevotella/*Porphyromonas*
Fusobacterium necrophorum

Less common or transients

Haemophilus influenzae
Neisseria meningitidis
Gram-negative rods

protected by the layer of mucus that lines the stomach. Organisms that are pH-susceptible and survive are generally protected by being enmeshed in food as they move to the small intestine. The stomach acidity reduces the number of viable organisms that reach the small intestine.

The small intestine contains fewer microorganisms compared with those usually present in the colon. Microorganisms prevalent in the colon may produce a count of 10^{12} bacteria per gram of solid material. In fact, the colon contains over 70% of all microbes found in the body. Obligate anaerobes, such as *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Prevotella*, and *Porphyromonas*, far outnumber the facultative gram-negative bacilli, making up more than 90% of the microbial biota of the large intestine. Gram-positive cocci belonging to the genera *Streptococcus* and *Enterococcus* and yeasts are also present in the large intestine. A single-layer epithelium, renewed every 5 days, lines the intestine. The tight junction spaces between epithelial cells make it difficult for bacteria to penetrate the epithelium. Many of the bacteria colonizing the intestines do so using pili.

The gastrointestinal tract population may be altered by antimicrobial agents. In some cases, certain populations or organisms are eradicated or suppressed, and other members of the indigenous biota can proliferate. For example, *C. difficile* or the yeast *Candida albicans* can flourish in the intestinal tract of some people who are taking an oral broad-spectrum antimicrobial agent. This alteration can be the cause of a severe necrotizing enterocolitis (*C. difficile*), diarrhea (*C. albicans*, *S. aureus*), or other superinfection. The bacteria constituting the usual intestinal biota also carry out various metabolic degradations and nutrient production that appear to play a role in the health of the host. The organisms found in the gastrointestinal tract are summarized in [Box 2.5](#).

Normal microbiota of the genitourinary tract

The kidneys, bladder, cervix, and fallopian tubes are normally sterile, although a few organisms originating from the perineum can be found in the distal urethra, particularly in women.

BOX 2.5 Microorganisms found in the gastrointestinal tract

Bacteroides spp.
Bifidobacterium spp.
Clostridium spp.
Enterobacterales
Enterococcus spp.
Eubacterium spp.
Fusobacterium spp.
Lactobacillus spp.
Peptostreptococcus spp.
Peptococcus spp.
Porphyromonas spp.
Prevotella spp.
Streptococcus spp.

Case check 2.2

In the Case in Point, antimicrobial therapy led to an alteration of the patient's normal gastrointestinal tract biota. As shown in [Box 2.5](#), *Clostridium* spp. and *C. difficile* can be part of the normal gastrointestinal tract microbiota in humans. In this case, eliminating some of the bacterial gut biota allowed *C. difficile* organisms to grow unchecked and cause an infection called *C. difficile*–associated disease that is often associated with prolonged antimicrobial therapy, as discussed in [Chapter 22](#).

The urethra is colonized in its outermost segment by organisms found on the skin. The composition of the vaginal microbiota is consistent with hormonal changes and age. Before puberty and in postmenopausal women, vaginal biota primarily consists of yeasts, gram-negative bacilli, and gram-positive cocci. During childbearing years, high estrogen levels promote the deposition of glycogen in vaginal epithelial cells. Lactobacilli metabolize glycogen from vaginal epithelial cells to maintain a low pH, creating an environment that is inhibitory to many organisms. However, the low pH encourages colonization of the vagina with lactobacilli, anaerobic gram-negative bacilli, and gram-positive cocci. Microorganisms usually isolated from the genitourinary tract are listed in [Box 2.6](#).

Role of the microbial biota in the pathogenesis of infectious disease

Some organisms that make up the microbial biota live off the host's nutrients, but in most cases, they provide some benefit to the host, creating a symbiotic relationship with the host, as mentioned earlier. However, certain members of the normal microbiota are **opportunists**; they cause disease when their habitat is altered or when the host's immune system is compromised. In the case of trauma, either accidental or surgical, enough of the normal microbiota found in the traumatized area may reach sterile or other areas in the body where these organisms are not part of the microbial biota. For example, patients who undergo surgery become susceptible to infections caused by organisms that colonize the surgical site (e.g., abdominal cavity); leakage following perforation of the colon spills the contents of the colon into the peritoneal cavity, leading to an infection by the colon biota.

BOX 2.6 Microorganisms found in the genitourinary tract**Common residents**

Bacteroides spp.
Clostridium spp.
Enterococcus spp.
Lactobacillus spp.
Peptostreptococcus spp.
Staphylococcus epidermidis
Diphtheroids (*Corynebacterium* spp.)

Less common or transients

Group B streptococci
Acinetobacter spp.
Candida albicans
Enterobacterales
Staphylococcus aureus

The host's immune response may be reduced or altered because of suppression by immunosuppressive drugs, chemotherapy, or radiation. Individuals with lymphoma, leukemia, or other blood disorders in which there is a functional defect in phagocytic activity or a decrease in the number of functioning cells or in which chemotactic activity is impaired also may have a reduced immune response. Members of the microbial biota also may initiate an infection or make an infection more serious in patients with chronic illnesses, including diabetes or severe hepatic disease such as cirrhosis.

Role of the microbial biota in the host defense against infectious disease

The microbial biota provide beneficial effects, and the development of immunologic competence depends on this biota. Vitamins and other essential nutrients are synthesized by certain bacteria in the intestine and appear to contribute to the overall health of the host.

The immune system is constantly primed by contact with microorganisms. In the gut, normal microbiota interact with host Toll-like receptors (TLRs), such as TLR5 found on epithelial cells and dendritic cells. Activation of TLR5 recruits T and B lymphocytes (T and B cells), leading to the production of immunoglobulin A (IgA) to limit the overcolonization of gut microbiota. Animals born and raised in a germ-free environment have a poorly functioning immune system. Exposure to otherwise innocuous organisms can be fatal to such animals. Likewise, consider how a sterile environment might affect a newborn. Antibody production would not be stimulated, and the mononuclear phagocyte system would remain undeveloped. Serum immunoglobulin G (IgG) and other antibodies effective against microorganisms would be suppressed, which would make the individual more susceptible to pathogenic microorganisms. Without activation by microorganisms and the supporting action of antigen-presenting cells and cytokines, cell-mediated immunity would not develop normally.

The microbial biota produce conditions at the microenvironmental level that block colonization by extraneous pathogens (e.g., competition for nutrients and secretion of bacteriocins). These proteins are produced by a variety of

gram-positive and gram-negative bacteria and appear to give the secreting bacterium an advantage because they can eliminate other bacteria that would compete for nutrients and space. Some species of bacteria produce metabolic by-products that result in a microenvironment that is hostile to potential pathogens.

When the composition of the indigenous biota is altered (e.g., by broad-spectrum antimicrobial therapy), other organisms capable of causing disease may fill the void. For example, gastroenteritis caused by *Salmonella* is generally not treated with antimicrobial agents and is better eliminated by natural exclusion by the colon biota. If the microbial biota are eliminated, such as in patients receiving antimicrobial therapy, resistant or more pathogenic species may be able to establish infection. For example, the yeast *C. albicans* can multiply and cause diarrhea or infections in the mouth or vagina when the normal biota have been eliminated.

The microbial biota play an important role in both health and disease. Although reduction of the usual biota can have profound negative effects, many common infections are caused by members of the resident biota. Knowledge of the role of these organisms in the pathogenesis of an infectious disease is helpful in assessing their significance when they are isolated from clinical samples.

Microbial factors contributing to pathogenesis and virulence

Pathogenesis

Pathogenicity is the ability of a microbe to produce disease in an individual. An organism may be described as a *true pathogen* or an *opportunistic pathogen*. True pathogens are organisms recognized to cause disease in healthy immunocompetent individuals a high percentage of the time. Bacterial species such as *Yersinia pestis* and *Bacillus anthracis* are pathogenic in nearly all situations; when these species are recovered in clinical samples, they are clinically significant.

Today, individuals with disease are living longer and are more likely to undergo invasive medical procedures, organ transplantation, and insertion of prosthetic devices, making them more susceptible to infections. As a result, organisms of the normal biota are being seen with increasing frequency in those patients and individuals who are immunosuppressed. *H. influenzae* colonizes the upper respiratory tract of healthy individuals without causing disease, but given the opportunity, it can rapidly produce a life-threatening infection involving the lower respiratory tract. Organisms such as *S. epidermidis* do not cause disease under usual conditions but can induce an infectious process in patients with prosthetic devices. *H. influenzae* and *S. epidermidis* are called *opportunistic pathogens*, and the infections they cause are called *opportunistic infections*. Table 2.1 lists common opportunistic microorganisms and the conditions with which they are commonly associated.

Because of compromised hosts, our definition of *pathogen* must be expanded to apply to virtually any microorganism when conditions for infection are met. In deciding whether a particular organism that has been isolated is a pathogen, we also must consider the human host from whom the organism was isolated and whether that host has underlying disease

Table 2.1 Abbreviated list of opportunistic microorganisms

Conditions compromising host defenses	Organism(s)
Foreign bodies (catheters, shunts, prosthetic heart valves)	<i>Staphylococcus epidermidis</i> <i>Cutibacterium acnes</i> Viridans streptococci <i>Serratia marcescens</i> <i>Pseudomonas aeruginosa</i> <i>Aspergillus</i> spp. <i>Candida albicans</i>
Alcoholism	<i>Streptococcus pneumoniae</i> <i>Klebsiella pneumoniae</i>
Burns	<i>Pseudomonas aeruginosa</i> <i>Acinetobacter baumannii-calcoaceticus</i> complex <i>Staphylococcus aureus</i>
Hematoproliferative disorders	<i>Cryptococcus neoformans</i> Varicella-zoster virus
Cystic fibrosis	<i>Pseudomonas aeruginosa</i> <i>Burkholderia cepacia</i>
Immunosuppression (drugs, congenital disease)	<i>Aspergillus</i> spp. <i>Candida albicans</i> <i>Pneumocystis jirovecii</i> Diphtheroids (<i>Corynebacterium</i> spp.) <i>Pseudomonas</i> spp. <i>Staphylococcus</i> spp. Cytomegalovirus Herpes simplex virus Varicella-zoster virus

that may increase susceptibility to infection. For example, the potential pathogen list for a healthy 20-year-old individual is much shorter than that for a healthy 90-year-old individual, a transplant recipient, or a 20-year-old individual with acquired immunodeficiency syndrome (AIDS). Almost any organism in the right place can cause an infection, and this must be considered when performing invasive procedures because an inadvertent result of intervention can be the transfer of an organism from where it is present as part of the indigenous biota to a place where it can replicate and cause infection. Contamination by resident skin biota of a surgical incision can lead to a serious wound infection.

An **iatrogenic infection** is an infection that occurs as the result of medical treatment or procedures. For example, many patients who have indwelling urinary catheters develop a urinary tract infection. Although placement of the catheter was a necessary procedure in the medical treatment of the individual, its use may result in an infection. Patients who are given immunosuppressive drugs because they have received an organ transplant are more susceptible to infection. Because any infection in such a patient would probably be the result of the physician-ordered drug therapy, it would be considered an iatrogenic infection.

Routes of transmission

The first step in initiating an infection is for the infectious agent to gain access to the host. The route by which a pathogen can be transmitted to a susceptible host is an important factor in the establishment of infection. The agent must be able to evade host defenses and colonize the tissue at the point of entry. Although some organisms may be naturally transmitted by more than one route, most have a preferred portal of entry. These routes can be characterized as through air (inhalation), via food and water (ingestion), through close contact (includes sexual transmission), through cuts and bites, and via arthropods; animal diseases that can infect humans are transmitted through animal contact (**zoonoses**). The routes of transmission are summarized in [Table 2.2](#). [Fig. 2.1](#) shows the routes of entry to and exit of microbes from the body.

Airborne transmission

Respiratory spread of infectious disease is common and is generally an efficient way to enter a host. Often, the respiratory secretions from an infected host are aerosolized by coughing, sneezing, and talking. Very small particles, referred to as *aerosols*, are the residue from the evaporation of fluid from larger droplets and are light enough to remain airborne for long periods. Large respiratory droplets fall to the ground before they evaporate contaminating surfaces. This results in droplet-contact spread in which disease transmission occurs because of touching the contaminated surface.

Pathogens that are spread through the air generally must be resistant to drying and inactivation by ultraviolet light. Some infectious agents may be transmitted by dust particles that have become airborne. As discussed earlier in this chapter, the body has many defenses against airborne infectious agents. The nasal turbinates, oropharynx, and larynx provide a twisting, mucus-lined passageway that makes direct access to the lower respiratory tract mechanically difficult. In addition, the lower portions of the respiratory tract contain ciliary epithelium that sweeps organisms upward. For a microorganism to cause disease, it must circumvent these defenses, penetrate the mucous layer, and attach to the epithelium. The host also produces secretory IgA, lysozyme, alveolar macrophages, and other factors that act on the pathogen that manages to get beyond the physical barriers.

Respiratory tract infections are the most common reason that patients of all ages seek medical attention. Although most upper respiratory tract infections are self-limiting and can be treated with over-the-counter medications, some are more serious. Streptococcal sore throat, sinusitis, otitis media, acute epiglottitis, and diphtheria can be serious and even life-threatening. Viral diseases causing the common cold and infectious mononucleosis are usually not life-threatening but can result in much discomfort and absenteeism from work or school.

Although all of the diseases mentioned can be spread via aerosols, some may also be transmitted via the fingers and hands; this is especially true of the common cold-causing rhinoviruses. The fingers and hands are contaminated with infectious nasal secretions because of hand-to-nose contact. The infectious viral particles are passed from the infected individual to a susceptible recipient via hand-to-hand or hand-to-face contact. The recipient transmits the virus picked up from

the infected individual by touching the face and nose. In this case, the disease is transmitted via the respiratory route but not in the normal, classic manner of respiratory transmission.

Transmission may also result from contact with inanimate objects contaminated with the infectious agent (**fomites**). For example, a doorknob is contaminated by the hand and fingers of an infected individual, and the virus is transmitted to a susceptible person's hand and fingers when that person opens the door. Control of such transmission is often as simple as frequent handwashing.

Infections of the lower respiratory tract are less common but more serious than infections of the upper respiratory tract. The organisms causing these infections have managed to bypass host defenses, or the host defenses have been compromised (e.g., by alcoholism, heavy smoking), allowing the pathogen access to the normally sterile, deeper portions of the respiratory tract.

The most common microorganism causing lower respiratory tract infection in individuals older than 30 years of age is *S. pneumoniae*. Risk increases with age. Although the pneumococcus is the most common cause of community-acquired pneumonia, it is also often seen in aspiration pneumonia, a common type of hospital-acquired pneumonia. Pneumococcal pneumonia begins suddenly and is a serious, life-threatening disease, particularly in older patients. The bacteria produce a large capsule that resists phagocytosis. In chronic lower respiratory tract infections, the survival of the infecting agent within phagocytes plays a role in the pathogenic mechanism. As the agent of tuberculosis, a chronic debilitating infection, *M. tuberculosis* is the classic example of an intracellular pathogen. This organism is highly virulent, is invasive, survives well, and multiplies within phagocytes. Infections are often chronic and result in granuloma formation.

Transmission by food and water

Transmission of gastrointestinal infections is usually a result of ingestion of contaminated food or water. In some situations, infection occurs via the fecal-oral route. The digestive tract is colonized with vast numbers of different microorganisms. Under usual conditions, the gut biota maintain a harmless relationship with the host. Gastric enzymes and acids in the stomach prevent survival of most organisms, but many survive and colonize the small intestine and colon.

Gastrointestinal infections result from organisms that survive the harsh conditions of the stomach and competition with the microbial biota and then produce damage to the tissues of the gastrointestinal tract. This damage is a result of either ingestion of a preformed toxin or disruption of the normal functioning of the intestinal cells by invasion of the pathogen or production of a toxin within the intestine.

Organisms that can cause disease by means of a preformed toxin, produced outside the body, include *Clostridium botulinum*, *Bacillus cereus*, and *S. aureus*. The severity of disease ranges from mild diarrhea to rapidly fatal intoxication. Food poisoning by *B. cereus* and *S. aureus* is relatively common and is self-limiting. Botulism, caused by *C. botulinum*, although rare, can be life-threatening.

Other bacteria produce a toxin after infecting the intestinal tract. Generally, to be effective as a pathogen, an organism must survive, adhere to, and colonize the intestinal mucosa and either produce a toxin or invade deeper tissues. A commonly seen cause of diarrhea and intestinal infection is

Table 2.2 Common routes of transmission^a

Route of exit	Route of transmission	Example
Respiratory	Aerosol inhalation	Influenza virus, tuberculosis, <i>Neisseria meningitidis</i>
	Nose or mouth (droplets) → hand or object → nose	Common cold (rhinovirus)
Salivary	Direct salivary transfer (e.g., kissing)	Oral-labial herpes; infectious mononucleosis
Gastrointestinal	Stool → hand → mouth and/or stool → object → mouth	Enteroviruses; hepatitis A
	Stool → water or food → mouth	Salmonellosis; shigellosis
Skin	Skin discharge → air → respiratory tract	Varicella; poxvirus infection
	Skin to skin	Human papillomavirus (warts); syphilis
Blood	Transfusion or needle prick	Hepatitis B; cytomegalovirus infection; malaria; HIV
	Insect bite	Malaria; relapsing fever; West Nile virus
Genital secretions	Urethral or cervical secretions	Gonorrhea; herpes simplex; <i>Chlamydia</i> infection
	Semen	Cytomegalovirus infection
Urine	Urine → hand → catheter	Hospital-acquired urinary tract infection
	Urine → aerosol (rare)	Tuberculosis
Eye	Conjunctival	Adenovirus
Zoonotic	Animal bite	Rabies, <i>Pasteurella multocida</i>
	Contact with carcasses	Tularemia
	Arthropod	Lyme disease, Rocky Mountain spotted fever, plague

^aThe examples cited are incomplete, and in some cases more than one route of transmission exists.

HIV, Human immunodeficiency virus.

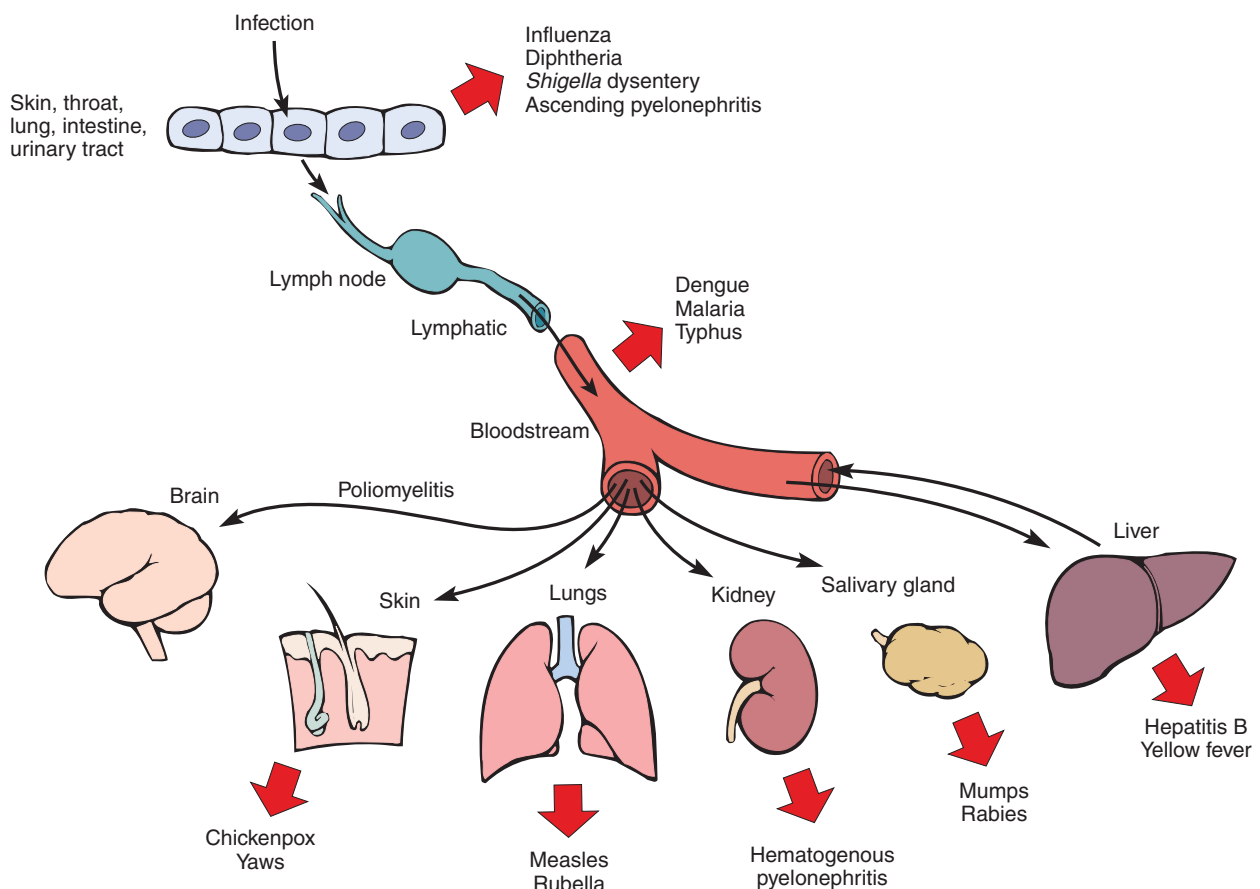


Fig. 2.1 Routes of entry and exit. (Reprinted from Mims, C.A., et al. (2001). *Mims' pathogenesis of infectious disease* [5th ed.]. San Diego: Elsevier Ltd. With permission from Elsevier.)

Escherichia coli. This organism is a member of the intestinal resident biota; however, some strains of *E. coli* produce virulence factors that cause alterations in the biochemical activity of the intestinal epithelial cells, resulting in problems with fluid and electrolyte control by the intestinal cells. These strains of *E. coli*, referred to as *diarrheagenic*, are a common cause of traveler's diarrhea and other intestinal problems. *Vibrio cholerae*, the cause of cholera, produces an enterotoxin that causes an outpouring of fluid from the cells into the lumen of the intestine. Massive amounts (20L per day) of fluid can be lost. Other intestinal pathogens include *C. difficile* (see Case in Point), *Shigella* spp., *Aeromonas hydrophila*, *Campylobacter jejuni*, and *Salmonella* spp. The infective dose, severity, and incidence of disease vary with the agent.

Numerous viruses also cause diarrheal disease. They multiply within the cells of the intestinal mucosa and affect the normal function of the cells. Viral agents in this category include rotavirus, adenovirus, coxsackievirus, and *Norovirus* spp. The incidence of diarrhea caused by these agents is high, especially when people are in close contact (e.g., in daycare centers, nursing homes, military camps, cruise ships). Some viruses enter via the gastrointestinal tract and infect the liver, such as hepatitis A and E viruses. Numerous parasites, such as *Cryptosporidium* spp., *Giardia lamblia*, *E. histolytica*, and *Balantidium coli*, also infect the gastrointestinal tract.

Close contact

Typically, the routes of transmission of infectious diseases require close contact. For a respiratory pathogen to be transmitted via aerosols, the susceptible host must be relatively close. However, aerosolized pathogens can stay airborne for some time and be carried by gentle air currents. For this discussion, *close contact* refers to passage of organisms by salivary, skin, and genital contact. Two prominent infections passed by direct transfer of saliva (e.g., kissing) are herpes simplex virus and Epstein-Barr virus. Skin-to-skin transfer of infectious disease is not as common as for some of the other routes, but diseases such as warts (human papillomavirus), syphilis, and impetigo result when material from infectious lesions inoculates the skin of a susceptible host. The list of sexually transmitted diseases is a long one. In North America, the most commonly transmitted venereal agents are human papillomavirus, *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, herpes simplex virus, *Treponema pallidum* subsp. *pallidum* (syphilis), *Trichomonas* spp., and human immunodeficiency virus (HIV).

Cuts and bites

The classic example of a bite-wound infection is rabies. However, human rabies is relatively rare. Of more concern with animal bites, and especially human bites, is infection by the mouth biota. Dog-bite and cat-bite infections often yield *Pasteurella multocida*, but the possibilities are extensive. Human bites are dangerous because the human oral biota comprises many different organisms in extremely high numbers, including obligate anaerobic bacteria.

Arthropods

Infectious agents can enlist the help of arthropods for transmission among hosts. Infection following a mosquito, tick,

flea, or mite bite is a common occurrence in many parts of the world. Diseases spread by arthropods include malaria, relapsing fever, plague, Rocky Mountain spotted fever, Lyme disease, West Nile fever, and untold numbers of regional viral hemorrhagic fevers. In most cases, the infectious agent multiplies in the arthropod, which then transmits the agent while feeding on a human host. This mode of transmission is much more common in the tropics because arthropods are active year-round. However, a significant number of **arthropod-borne** diseases occur in temperate zones.

Zoonoses

The route of transmission known as *zoonosis* depends on contact with animals or animal products. Certain organisms causing disease in animals may also infect humans who have contact with them. These diseases may be passed by animal bites (rabies), arthropod vectors (plague), contact with secretions (brucellosis), and contact with animal carcasses and products (tularemia, listeriosis). The diseases are transmitted by the routes already discussed. The common factor is that, regardless of the route, the disease is a disease of animals that is transmitted to humans. A partial list of zoonotic diseases and infecting organisms is provided in [Table 2.3](#).

Table 2.3 Zoonoses

Disease	Organism
Anthrax	<i>Bacillus anthracis</i>
Brucellosis	<i>Brucella</i> spp.
Erysipeloid	<i>Erysipelothrix rhusiopathiae</i>
Leptospirosis	<i>Leptospira interrogans</i>
Tularemia	<i>Francisella tularensis</i>
Ringworm	<i>Trichophyton</i> spp. <i>Microsporum</i> spp.
Lyme disease	<i>Borrelia burgdorferi</i>
Plague	<i>Yersinia pestis</i>
Rocky Mountain spotted fever	<i>Rickettsia rickettsii</i>
Yellow fever	Flavivirus
Encephalitis	Alphavirus
Rabies	Rhabdovirus
Blastomycosis	<i>Blastomyces dermatitidis</i>
Tuberculosis	<i>Mycobacterium bovis</i>
Q fever	<i>Coxiella burnetii</i>
Ornithosis	<i>Chlamydophila psittaci</i>
Gastroenteritis	<i>Campylobacter</i> spp. <i>Salmonella</i> spp.
Listeriosis	<i>Listeria monocytogenes</i>
Giardiasis	<i>Giardia lamblia</i>
Toxoplasmosis	<i>Toxoplasma gondii</i>
Tapeworms	<i>Taenia saginata</i> <i>Taenia solium</i> <i>Diphyllbothrium latum</i> <i>Echinococcus</i> spp.
Trichinosis	<i>Trichinella spiralis</i>

Virulence

Virulence is the relative ability of a microorganism to cause disease or the degree of pathogenicity. It is usually measured by the numbers of microorganisms necessary to cause infection in the host. Organisms that can establish infection with a relatively low infective dose are considered more virulent than organisms that require high numbers for infection. For example, because *Shigella* spp. cause disease with a relatively low infective dose (100 organisms), *Shigella* is considered a highly virulent organism. This generalization is misleading because the severity of disease caused by different organisms varies from one to another. If a microorganism requires a relatively high infective dose but the disease it causes is often fatal, we tend to think of the microorganism as highly virulent. A different organism may require a low infective dose but produce a relatively mild disease.

Microbial virulence factors

Infectious organisms have a wide variety of mechanisms or virulence factors that allow them to persist in a host and cause disease. Some virulence factors, such as capsules and toxins, are used by many organisms. Other virulence factors tend to be specialized and specific to one particular organism, such as the tissue tropism of *N. gonorrhoeae*. Virulence factors allow the pathogen to evade or overcome host defenses and cause disease, and they encompass functions such as facilitating adhesion to host cells or tissues, inhibiting phagocytosis, enhancing intracellular survival after phagocytosis, and damaging tissue through the production of toxins and extracellular enzymes. Many virulence factors are well defined, such as the diphtheria and cholera toxins, the capsule of *S. pneumoniae*, and the fimbriae of *N. gonorrhoeae*. Certain microorganisms produce extracellular factors that appear to aid in infection, but the exact role of these factors is unknown.

Ability to resist phagocytosis

Phagocytes, or phagocytic cells, such as macrophages and polymorphonuclear cells, play a major role in defending the host from microbial invasion. These cells ingest bacteria and destroy them. The lack of functioning phagocytic cells leaves the host susceptible to overwhelming infection. Therefore an extremely important event in the life of an invading pathogen is avoiding phagocytosis. There are many ways by which microbial species evade phagocytosis; some are listed in [Table 2.4](#).

The most common mechanism for evading phagocytosis used by microorganisms is a polysaccharide capsule. Many microorganisms possessing a capsule are highly virulent until removal of the capsule, at which point virulence becomes extremely low. Encapsulated strains of *S. pneumoniae* and *H. influenzae* are associated with highly invasive infections and are known to be more virulent than non-encapsulated strains. Host antibodies directed against the capsule can enhance phagocytosis and also decrease the microbe's virulence, a process called *opsonization*. The capsule is usually composed of polysaccharides but can also be made of proteins or a combination of proteins and carbohydrates. The capsule inhibits phagocytosis primarily by masking the cell surface structures that are recognized by receptors on the surface of the

phagocytic cell and in the same manner inhibits activation of complement by masking structures to which complement proteins bind.

Another bacterial structure that protects organisms from phagocytosis is protein A. Protein A in the cell wall of *S. aureus* helps the organism avoid phagocytosis by interfering with the binding of the host's antibodies to the surface of the organism. Antibodies bind to antigens via their fragment antigen-binding (Fab) portion. Protein A binds to the Fc (crystalline fragment) portion of IgG (at the opposite end of the Fab sites), preventing opsonization and phagocytosis by turning the antibody around on the surface.

Some organisms evade phagocytic cell killing by releasing toxins in tissues that kill phagocytes. Streptococci produce hemolysins that lyse red blood cells and induce toxic effects on white blood cells and macrophages. Pathogenic staphylococci release **leukocidins** that cause lysosomal discharge of white blood cells into the cytoplasm. A staphylococcal leukocidin, called *Panton-Valentine leukocidin*, is lethal to leukocytes and may contribute to the invasiveness of the organism. Other organisms inhibit chemotaxis, which is the movement of white blood cells to sites of tissue damage, and the host is less able to direct polymorphonuclear neutrophils (PMNs) and macrophages into the site of infection.

Structures that promote adhesion to host cells and tissues

Most infectious agents must attach to host cells before infection occurs. In intoxication diseases caused by exotoxins (e.g., botulism, staphylococcal food poisoning), microbial adherence is not important. However, in virtually all other cases, the bacterium, virus, or fungus requires adherence to host cells before infection and disease can progress. Without adhering to host cells, the pathogen can be carried away in fluid (e.g., urine or mucus). The microbial or viral surface structures that mediate attachment are called **adhesins**. Host cells must possess the necessary **receptors** for the adhesins, generally a carbohydrate. If the host or the infectious agent undergoes a mutation that changes the structure of the adhesin or the host receptor, adherence can be less efficient or not take place, and the virulence of the infectious agent is affected. [Fig. 2.2](#) illustrates surface bacterial structures that are involved in the pathogenesis of disease.

The main adhesins in bacteria are surface proteins and polysaccharides. Extending from the surface of many human bacterial pathogens are **fimbriae (pili)**. On the tips of fimbriae are adhesion proteins that bind to receptors on cell surfaces. Fimbriae enable bacteria to adhere to host cell surfaces, increasing the organism's colonizing ability and providing resistance to phagocytosis. Once bacteria are attached to nonphagocytic cells, phagocytosis by white blood cells is less likely to occur. For example, the strains of *E. coli* that cause traveler's diarrhea use their fimbriae to adhere to cells of the small intestine, where they secrete a toxin that causes the disease symptoms. Similarly, fimbriae are essential for *N. gonorrhoeae* to infect the epithelial cells of the genitourinary tract. Antibodies to the fimbriae of *N. gonorrhoeae* are protective by preventing the organism from attaching to the epithelial cells. Similarly, antibodies to viral adhesions prevent infection.

Table 2.4 Microbial interference with phagocytic activities

Microorganisms ^a	Type of interference ^b	Mechanism (or responsible factor)
<i>Streptococcus pyogenes</i>	Kill phagocyte Inhibit neutrophil chemotaxis Resist phagocytosis	Streptolysin induces lysosomal discharge into cell cytoplasm Streptolysin M and M-like proteins Hyaluronic acid capsule
<i>Staphylococcus aureus</i>	Kill phagocyte Inhibit opsonized phagocytosis Resist killing Block chemotaxis	Leucocidin induces lysosomal discharge into cell cytoplasm SpA binds Fc portion of Ab Polysaccharide capsule in some strains
<i>Bacillus anthracis</i>	Kill phagocyte Resist killing	Lethal factor of tripartite toxin Capsular polyglutamic acid
<i>Haemophilus influenzae</i>	Resist phagocytosis (unless Ab present)	Polysaccharide capsule
<i>Streptococcus pneumoniae</i>	Resist phagocytosis (unless Ab present)	Polysaccharide capsule
<i>Klebsiella pneumoniae</i>	Resist phagocytosis	Polysaccharide capsule
<i>Pseudomonas aeruginosa</i>	Kill phagocyte Resist phagocytosis	Exotoxin A kills macrophages; also four type III toxins: exoenzyme (Exo) Alginate exopolysaccharide
<i>Escherichia coli</i>	Resist phagocytosis (unless Ab present) Kill macrophages	O antigen (smooth strains) K antigen (acid polysaccharide)
<i>Salmonella</i> spp.	Resist phagocytosis (unless Ab present) Resist killing; survival in macrophages Kill phagocyte	Vi antigen Secreted products of SPI-2 Secreted products of SPI-1
<i>Clostridium perfringens</i>	Inhibit chemotaxis Resist phagocytosis	α -toxin Capsule
<i>Cryptococcus neoformans</i>	Resist phagocytosis	Capsular polyuronic acid
<i>Yersinia pestis</i>	Kill phagocyte	Yersinia outer protein (Yop)
<i>Mycobacteria</i>	Resist killing Digestion Inhibit lysosomal fusion	Cell wall substance
<i>Brucella abortus</i>	Resist killing	Cell wall substance
<i>Toxoplasma gondii</i>	Inhibit attachment to neutrophil Inhibit lysosomal fusion	

Ab, Antibody; SpA, staphylococcal protein A; SPI-1, *Salmonella* pathogenicity island 1; SPI-2, *Salmonella* pathogenicity island 2; Yop, *Yersinia* outer protein.

^aOften only the virulent strains show the type of interference listed.

^bSometimes the type of interference listed has been described only in a particular type of phagocyte (polymorph or macrophage) from a particular host, but it generally bears a relationship to pathogenicity in that host.

Modified from Mims, C. A. (2015). *The pathogenesis of infectious disease* (6th ed.). New York: Academic Press.

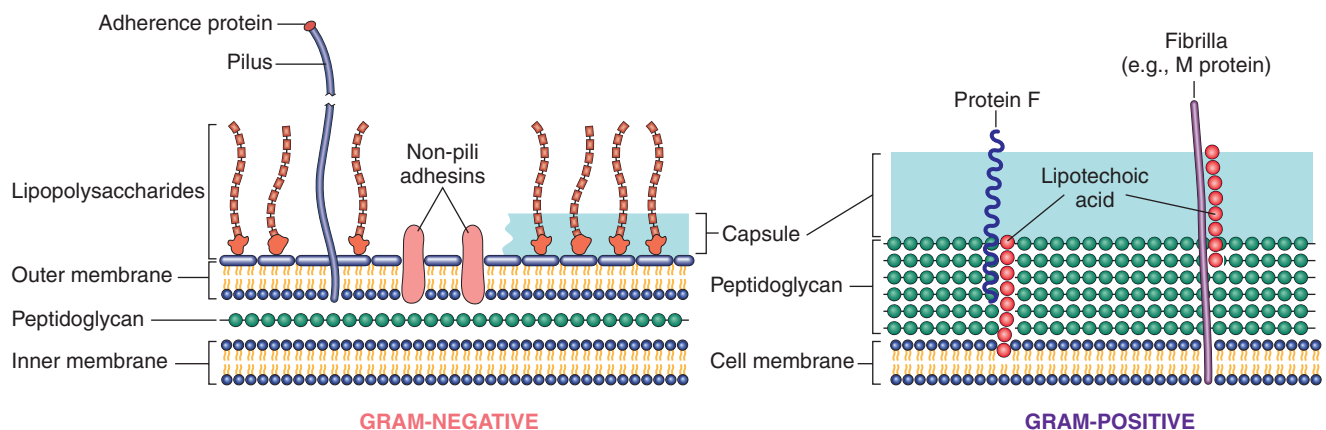


Fig. 2.2 Surface bacterial structures that are involved in the pathogenesis of disease. (From Kumar, V, et al. (2005). *Robbins and Cotran pathologic basis of disease* [7th ed.]. Philadelphia: Saunders.)

Some human pathogens express adhesion proteins not associated with fimbriae. When encountering host tissue, several gram-positive bacteria, like *S. aureus*, use molecular adhesion molecules that include teichoic acid, lipoteichoic acid, and lipoproteins. The primary tissue receptor is TLR2 found on epithelial cells, endothelial cells, and leukocytes. However, binding to TLR2 on keratinocytes leads to the release of antimicrobial peptides and proinflammatory cytokines and neutrophil-attracting chemokines, an integral part of our innate defense mechanism. An additional adherence mechanism used by bacteria is a biofilm. Some bacteria secrete a sticky polysaccharide that binds bacterial cells together and to host cells or nonliving surfaces. Biofilms, discussed in [Chapter 31](#), can also protect bacteria from phagocytosis and antibodies.

Ability to survive intracellularly and proliferate

Bacteria are engulfed by some host cells, such as macrophages, into a cytoplasmic vacuole called a *phagosome*. Phagosomes fuse with lysosomes producing phagolysosomes, resulting in microbial killing. Lysosomes contain several antimicrobial factors. The process of phagocytosis is discussed in more detail later in this chapter. After phagocytosis, some pathogens survive within the phagocytic cell. Microorganisms have developed several methods to prevent being killed intracellularly. Some organisms prevent fusion of phagosomes and lysosomes, others escape from the phagosome into the cytoplasm, and still others are resistant to the effects of the lysosomal contents.

Bacteria that prevent phagolysosomal formation can survive and multiply inside the macrophage. Bacterial species, such as *Chlamydia*, *Mycobacterium*, *Brucella*, and *Listeria* species, are easily engulfed by macrophages and phagocytes. However, these species are not only able to grow inside the macrophages, but they are protected from the host's other immune defenses and are called *intracellular parasites*. The bacterium *Coxiella burnetii* can grow inside the harsh environment of a phagolysosome.

To establish itself and cause disease, a pathogen must be able to replicate after attachment to host cells. Numerous host factors work to prevent proliferation: secretory antibody acts as an opsonin, enhancing phagocytosis; **lactoferrin** binds iron (an essential trace element); and **lysozyme** lyses bacteria. To be successful in establishing infection, infectious agents must be able to avoid or overcome these local factors. Lactoferrin competes with bacteria for free iron; *N. meningitidis* can use lactoferrin as a source of iron and is therefore not inhibited by its presence. The nonpathogenic *Neisseria* spp. are usually unable to use the iron in lactoferrin and are inhibited by its presence.

Several pathogens (*H. influenzae*, *N. gonorrhoeae*, and *N. meningitidis*) produce an IgA protease that degrades IgA found at mucosal surfaces. Other pathogens (influenza virus, *Borrelia* spp.) circumvent host antibodies by changing key surface antigens. The host produces antibodies against the "old" antigens, which are no longer effective because the agent now has "new" antigens that do not bind to antibodies made against the old antigens.

Pathogens exhibit an ability to penetrate and grow in tissues, a process called *invasion*. With some organisms, the invasion is localized and involves only a few layers of cells. For example, *N. gonorrhoeae* attaches to cells in the genital

area, penetrates into the cells, and multiplies. The bacteria pass through the cells into the subepithelial space and establish an infection. With some organisms, such as *Salmonella* spp., the organisms spread to distant sites (organs and tissues), often via the bloodstream. This is called **dissemination**. Other organisms, such as *Corynebacterium diphtheriae*, do not spread beyond their initial site of infection, yet the disease they produce is serious and often fatal because the exotoxins they produce are disseminated. Certain organisms that survive phagocytosis can be disseminated rapidly to many body sites, but the organisms themselves are not invasive. The phagocyte carries the organism to other body sites, but the bacterium itself is incapable of penetrating tissues.

Ability to produce extracellular toxins and enzymes

Generally, disease from infection is noticeable only if tissue damage occurs. This damage may be from toxins, either **exotoxins** or **endotoxins**, or from inflammatory substances that cause host-driven, immunologically mediated damage. The ability of organisms to produce exotoxins and extracellular enzymes is another major factor that contributes to the virulence and invasiveness of organisms. Toxins are poisonous substances produced by organisms that interact with host cells, disrupting normal metabolism and causing harm. Exotoxins are produced by both gram-negative and gram-positive bacteria and are secreted by the organism into the extracellular environment, or they are released on lysis of the organism.

Exotoxins can mediate direct spread of the microorganisms through the matrix of connective tissues and can cause cell and tissue damage. Some organisms produce soluble substances, such as proteases and hyaluronidases that liquefy the hyaluronic acid of the connective tissue matrix, helping bacteria to spread in tissues, promoting the dissemination of infection.

Endotoxins are a constituent, the lipopolysaccharide (LPS), of the outer cell membrane of gram-negative bacteria exclusively. Endotoxins, in contrast with exotoxins, do not have enzyme activity, are secreted in only very small amounts, do not have specificity in their activity on host cells, are generally not very potent, and are not destroyed by heating. Endotoxins are released in large amounts when the bacterial cell lyses.

Exotoxins

Most bacterial exotoxins are composed of two subunits: one is nontoxic and binds the toxin to the host cells and the other has toxic activity. The toxin gene is commonly encoded by phages, plasmids, or transposons. Only the organisms that carry the DNA coding for the toxin gene produce toxin. Isolates of *C. difficile*, for example, must be tested for the presence of toxin genes, such as in the Case in Point. Diphtheria toxin inhibits protein synthesis and affects the heart, nerve tissue, and liver. Botulinum toxin is a neurotoxin that blocks nerve impulse transmission, causing flaccid paralysis, especially in infants. *S. pyogenes* and *S. aureus* both produce exfoliatin, which causes a rash and massive skin peeling or exfoliation. [Table 2.5](#) lists many bacterial exotoxins that are important in disease.

Table 2.5 Examples of exotoxins of pathogenic bacteria

Bacterium	Disease caused in humans	Toxins
<i>Bacillus anthracis</i>	Anthrax	Lethal, edema-producing toxins
<i>Bordetella pertussis</i>	Whooping cough (pertussis)	Lethal, necrotizing toxin
<i>Clostridium botulinum</i>	Botulism	Six type-specific lethal neurotoxins ^a
<i>Clostridioides difficile</i>	Antibiotic-associated diarrhea, pseudomembranous colitis	Toxin A, enterotoxin ^a Toxin B, cytotoxin ^a
<i>Clostridium novyi</i>	Gas gangrene	Alpha, lethal, necrotizing Beta, lethal, necrotizing, hemolytic Gamma, lethal, necrotizing, hemolytic Delta, hemolytic Epsilon, lethal, hemolytic Zeta, hemolytic
<i>Clostridium perfringens</i>	Gas gangrene, food poisoning, enteritis necroticans	Alpha, lethal, necrotizing, hemolytic ^a Beta, lethal, necrotizing Gamma, lethal, necrotizing, hemolytic Delta, lethal Epsilon, lethal, necrotizing Iota, lethal, necrotizing Theta, lethal, cardiotoxic, hemolytic Kappa, lethal, proteolytic Enterotoxin ^a
<i>Clostridium septicum</i>	Gas gangrene	Alpha, lethal, hemolytic, necrotizing
<i>Clostridium sordellii</i>	Gas gangrene	Lethal toxin ^a Hemorrhagic toxin ^a
<i>Clostridium tetani</i>	Tetanus	Tetanospasmin, lethal, neurotoxic ^a Neurotoxin, nonspasmogenic Tetanolysin, lethal, cardiotoxic, hemolytic
<i>Corynebacterium diphtheriae</i>	Diphtheria	Diphtheria toxin, lethal, necrotizing ^a
<i>Escherichia coli</i> (unique strains)	Diarrhea	Heat-labile enterotoxin ^a Heat-stable enterotoxin Shiga toxin
<i>Pseudomonas aeruginosa</i>	Pyogenic infections	Exotoxin A
<i>Staphylococcus aureus</i>	Pyogenic infections, enterotoxemia	Alpha, lethal, necrotizing, hemolytic Beta, lethal, hemolytic Gamma, lethal, hemolytic Delta, hemolytic Exfoliating toxin ^a Enterotoxin ^a
<i>Streptococcus pyogenes</i>	Pyogenic infections, scarlet fever, rheumatic fever	Erythrogenic, nonlethal Streptolysin O, lethal, hemolytic, cardiotoxic Streptolysin S, lethal, hemolytic
<i>Vibrio cholerae</i>	Cholera	Cholera toxin, lethal, enterotoxic ^a
<i>Salmonella Typhimurium</i>	Enteritis	Enterotoxin? ^a
<i>Shigella</i> spp.	Dysentery	Enterotoxin ^a
<i>Yersinia pestis</i>	Plague	Murine toxin, cytotoxic pore-forming toxins ^a

^aToxins that produce harmful effects of infectious disease.

Endotoxins

Endotoxins are composed of the LPS portion of the outer membrane on the cell wall of gram-negative bacteria. The cell wall of gram-negative microorganisms is composed of two layers—the inner peptidoglycan layer and an outer membrane. The LPS is contained in the outer membrane along with proteins and phospholipids (see Fig. 2.2). LPS contains three regions: an inner lipid A, a core polysaccharide, and an antigenic oligosaccharide (O region). Lipid A is hydrophobic and anchors LPS in the outer membrane, while the hydrophilic O-region extends a short distance from the cell.

The lipid A portion of LPS is responsible for the toxic activity of endotoxin. Its chemical structure varies among gram-negative bacteria, making some species more virulent than others. LPS stimulates the release of proinflammatory cytokines (e.g., tumor necrosis factor and interleukin 1), chemokines, and other inflammatory mediators that aid in mounting an innate immune response. These are the chemical mediators that produce the effects of endotoxin that consist of dramatic changes in blood pressure, clotting, body temperature, circulating blood cells, metabolism, humoral immunity, and cellular immunity.

Endotoxins stimulate the fever centers in the hypothalamus, increasing body temperature within 1 hour after exposure. Endotoxins can produce severe hypotension within 30 minutes. Septic or endotoxic shock is a serious and potentially life-threatening condition. In contrast with shock caused by fluid loss, such as shock seen in severe bleeding, septic shock is unaffected by fluid administration. Endotoxins also initiate coagulation, which can result in disseminated intravascular coagulation. This process depletes clotting factors and activates fibrinolysis so fibrin-split products accumulate in the blood. These fragments are anticoagulants and can cause serious bleeding. Another feature of patients with endotoxic shock is severe neutropenia, which can occur within minutes after exposure. It results from sequestration of neutrophils in capillaries of the lung and other organs. Leukocytosis follows neutropenia because neutrophils are released from the bone marrow.

Endotoxins also produce a wide variety of effects on the immune system. They stimulate proliferation of B lymphocytes in some animal species, activate macrophages, activate complement, and have an adjuvant effect with protein antigens. They also stimulate interferon production and cause changes in carbohydrate and lipid metabolism as well as sensitivity to epinephrine. A severe infection with gram-negative bacteria can lead to serious and often life-threatening situations. Bacterial exotoxins and endotoxins are compared in Table 2.6.

Case check 2.3

In the Case in Point, diarrhea caused by *C. difficile* was diagnosed in a patient who had been treated with antimicrobial therapy for several days. *C. difficile* produces potent virulence factors, including an enterotoxin called *toxin A* and a cytotoxin called *toxin B*. *C. difficile* is a significant cause of diarrhea in patients who have received prolonged courses of antimicrobial therapy in both outpatient and inpatient settings.

Table 2.6 Differences between bacterial exotoxins and endotoxins

Characteristic	Exotoxins	Endotoxins
Organism type	Gram-positive and gram-negative	Gram-negative
Chemical nature	Simple protein	Protein-lipid-polysaccharide
Stability to heating (100°C)	Labile	Stable
Detoxification by formaldehyde	Detoxified	Not detoxified
Neutralization by homologous antibody	Complete	Partial
Biological activity	Individual to toxin	Same for all toxins

Host resistance factors

Physical barriers

Humans have evolved a complex system of defense mechanisms to prevent infectious agents from gaining access to and replicating in the body. Healthy skin is an effective barrier against infection. The stratified and cornified epithelium presents a physical barrier to penetration by most microorganisms. Only a few microorganisms can enter the body by way of intact skin. Some of these microorganisms and others that normally enter when the skin barrier is compromised are listed in Table 2.7. Most of the organisms listed in Table 2.7 require help in penetrating the skin barrier (e.g., animal or arthropod bite) or microscopic tears. Healthy, intact skin is clearly the primary mechanical barrier to infection. The skin also has substantial numbers of microbial biota that are usually nonpathogenic organisms that contribute to a low pH by their metabolism, compete for nutrients, and produce bactericidal substances (e.g., bacteriocins). In addition, long-chain fatty acids secreted by sebaceous glands are directly toxic to some bacteria and contribute to an acid pH ensuring that relatively few organisms can survive and prosper in the acidic environment of the skin. These conditions prevent colonization by transient, possibly pathogenic organisms.

Because mucous membranes interact with the environment, they are more easily penetrated by pathogenic agents. However, mucous membranes are covered by mucus, a viscous mixture of mucin, glycoproteins, antibodies (primarily IgA), and other antibacterial factors. For a pathogen to colonize mucous membranes and potentially cause an infection, it must overcome these factors. In addition, the epithelium lining mucous membranes is periodically shed, taking away any bacteria attached to the cells. Underlying the mucous membranes are several cells of the immune system including macrophages, dendritic cells, T and B lymphocytes, and innate lymphoid cells.

Cleansing mechanisms

Normally, the term *cleansing* suggests a liquid. However, one of the most effective cleansing mechanisms humans have is

Table 2.7 Microorganisms that infect skin or enter the body via the skin

Microorganisms	Disease	Comments
Arthropod-borne viruses	Various fevers, encephalitides	150 distinct viruses, transmitted by infected arthropod bites
Rabies virus	Rabies	Bites from infected animals
Rickettsiaceae	Typhus, spotted fevers	Bites from infected arthropods
<i>Leptospira</i>	Leptospirosis	Contact with water containing infected animal urine
<i>Staphylococcus aureus</i>	Boils, impetigo	Most common skin invaders
<i>Streptococcus pyogenes</i>	Impetigo, erysipelas	
<i>Bacillus anthracis</i>	Cutaneous anthrax	Systemic disease following local lesion at inoculation site
<i>Treponema pallidum</i> subsp. <i>pallidum</i>	Syphilis	Warm, moist skin is more susceptible
<i>Yersinia pestis</i>	Plague	Bite from infected rat flea
<i>Plasmodium</i> spp.	Malaria	Bite from infected mosquito
Dermatophytes	Ringworm, athlete's foot	Infection restricted to skin, nails, and hair

the desquamation of the skin surface. The keratinized squamous epithelium or outer layer of skin is being shed continuously. Many of the microorganisms colonizing the skin are disposed of with the sloughing of the epithelium.

More obvious is the cleansing action of the fluids of the eye and the respiratory, digestive, urinary, and genital tracts. The eye is continually exposed to microorganisms, which means this organ has some highly developed antimicrobial mechanisms. Tears bathing the cornea and sclera not only lubricate the eye but also wash foreign matter and infectious agents away from the surface. Additionally, tears contain IgA and lysozyme.

The respiratory tract is also continuously exposed to microorganisms and is protected by nasal hairs, ciliary epithelium, and mucous membranes. A continuous flow of mucus emanates from the membranes lining the nasopharynx, which traps particles and microbes and sweeps them to the oropharynx, where they are either expectorated or swallowed. The trachea is lined with ciliary epithelium. These cells have hairlike extensions (cilia) that sweep particles and organisms upward toward the oropharynx. This material is expectorated or swallowed. Heavy smokers have a significant reduction in ciliated epithelial cells and therefore are more susceptible to respiratory infections. The purpose of these mechanisms is to prevent infectious agents and other particles from reaching the bronchioles and lungs. Under normal conditions, they are very effective, and the air moving into and out of the lungs is sterile.

Bacteria are swallowed into the gastrointestinal tract either as part of the mouth biota and upper respiratory tract or in liquids and food. Most bacteria are destroyed by the low pH

found in the stomach. However, some bacteria can survive and pass into the small intestine. The number of bacteria in the intestine increases as the distance from the stomach increases. The distal portion of the colon contains the highest number of organisms. The gastrointestinal tract is protected by mucous secretions and peristalsis that prevent the organisms from attaching to the intestinal epithelium.

The genitourinary tract is cleansed by voiding urine. Consequently, only the distal portion of the urethra typically has a microbial population. The vagina contains a large population of organisms as part of the indigenous biota. The acidity of the vagina, resulting from the breakdown of glycogen by the resident biota, tends to inhibit transient organisms from colonizing. The cervix is normally sterile.

Antimicrobial substances

Various substances produced in the human host have antimicrobial activity. Some are produced as part of the phagocytic defense and are discussed later. Others, such as fatty acids, hydrogen chloride in the stomach, lysozyme, and secretory IgA have already been mentioned.

Lysozyme, a low-molecular-weight (approximately 14 kDa) enzyme found throughout the animal kingdom, hydrolyzes the peptidoglycan layer of bacterial cell walls causing lysis. In some bacteria, the peptidoglycan layer is directly accessible to lysozyme. These bacteria are killed by the enzyme alone. In other bacteria, the peptidoglycan layer is exposed after other agents have damaged the cell wall (e.g., antibody and complement, hydrogen peroxide). In these cases, lysozyme acts with the other agents to cause death of the infecting bacteria. Through evolution, some bacteria exhibit chemical modification of peptidoglycan, increasing resistance to lysozyme. In fact, *S. aureus* is nearly totally resistant to its effects. Lysozyme is found in serum, mucus, tissue fluids, tears, breast milk, saliva, and sweat.

The **complement system** is composed of about 20 proteins, mostly produced by the liver. Many of the proteins are proenzymes, released in an inactive form but ultimately metabolized into an active enzyme. During metabolism, the proenzymes are cleaved into an "a" and "b" component, each with different functions. The outcomes of complement activation are production of opsonins, chemotaxins, and other inflammatory functions. In addition, the proteins form a membrane attack complex that can lyse plasma membranes, such as those of bacteria.

Antibodies, especially secretory IgA, are found in saliva and mucous secretions of the respiratory, genital, and digestive tracts. They may serve as **opsonins**, enhancing phagocytosis, or they may fix complement and neutralize the infecting organism. Serum also contains low-molecular-weight cationic proteins, termed β -*lysins*. These proteins are lethal against gram-positive bacteria and are released from platelets during coagulation. The site of action is the cytoplasmic membrane. These antimicrobial substances and systems work best together. A combination of antibody, complement, and lysozyme is significantly more effective in killing bacteria than each alone or than any combination in which one or more are missing.

Interferons inhibit viral replication and are arguably the most important initial mechanism to control viral infections. Interferons are a group of cellular proteins induced by

eukaryotic cells in response to viral infection or other inducers. Interferons play a significant role in the innate response against foreign invaders. Interferons bind to interferon receptors on cells and produce an antiviral state by activating antiviral genes in both infected and uninfected cells. These gene products can block viral penetration and inhibit viral replication. Uninfected cells that have been exposed to interferon are refractory to virus infection. Interferons also activate macrophages and natural killer cells, enhancing their antiviral activity. Some interferons play a role in the adaptive immune response by stimulating dendritic cells—which are antigen-presenting cells—and T cells. Numerous bacteria, viruses, and their products induce interferon production. Interferon is so named because it can interfere with viral replication.

There are instances in which viruses escape the effects of interferons, either because they are resistant to the antiviral effects or because the induction of interferon in the host does not take place. For example, the vaccinia virus can resist the effects of interferons by inactivating interferon gamma. Other viruses can produce persistent infections because these viruses do not induce interferon production.

Phagocytosis

Phagocytosis is an essential component in the resistance of the host to infectious agents. It is the primary mechanism in the host defense against extracellular bacteria and numerous viruses and fungi. The PMNs, macrophages, and monocytes are the body's second line of defense.

The stem cells for neutrophils arise in the bone marrow, where they differentiate to form mature neutrophils. During this maturation, the cells synthesize myeloperoxidase, proteases, cathepsin, lactoferrin, lysozyme, and elastase. These products are incorporated into membrane-bound vesicles called *lysosomes*. The lysosomes appear as azurophilic granules on Wright's stain and contain enzymes, oxygen-reactive molecules, and other substances necessary for the killing and digestion of engulfed particles. The PMN also has receptors on the cell membrane for some complement components that stimulate cell migration, the metabolic burst, and secretion of the lysosome contents into a phagosome. The PMN, an end-stage cell, has a circulating half-life of 2 to 7 hours and makes up the majority of white blood cells in circulation of healthy individuals. It can migrate to the tissues, where its half-life is less than 1 week.

Macrophages also originate in the bone marrow from stem cells. They circulate as monocytes for 1 to 2 days and then migrate through the blood vessel walls into the tissues and reside in specific tissues as part of the mononuclear phagocyte system. These cells are widely distributed in the body and play a central role in specific immunity and nonspecific phagocytosis (Table 2.8).

Chemotaxis

Four activities must occur for phagocytosis to take place: (1) migration of the phagocyte to the area of infection (chemotaxis), (2) attachment of the particle to the phagocyte, (3) ingestion, and (4) killing. The PMNs circulate through the body followed by movement into the tissues, an action called **diapedesis**, which is the movement of the neutrophils

Table 2.8 Tissue distribution of monocytes/macrophages

Cell name	Tissue distribution
Monocyte	Blood
Kupfer cell	Liver
Alveolar macrophage	Lung
Histiocyte	Connective tissue
Microglial cell	Central nervous system
Mesangial cell	Kidney
Macrophage	Spleen, lymph nodes

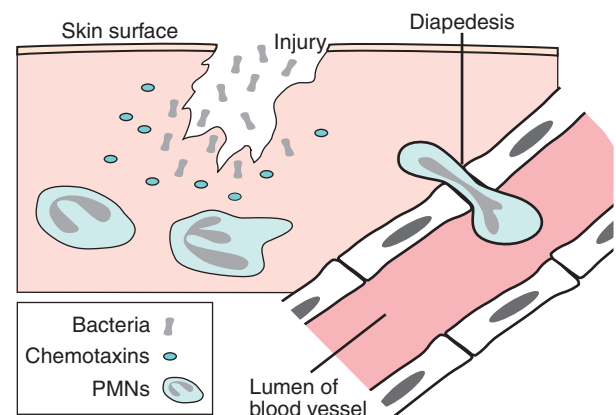


Fig. 2.3 Phagocytosis: chemotaxis migration of phagocytes PMNs, Polymorphonuclear neutrophils.

between the endothelial cells of the blood vessels into the tissues. The body is under constant surveillance by these and other phagocytic cells. When an infection occurs, massive numbers of PMNs accumulate at the site. This accumulation is not a random event; rather, it is a directed migration of PMNs into the area needing their services. This migratory process is called **chemotaxis**. Several substances serve as **chemotactic agents** (chemotaxins), which attract phagocytic cells. These include certain complement proteins, several bacterial products, products from damaged tissue cells, and products from responding immune cells. As the organism triggers tissue damage and inflammation, chemotaxis of phagocytes occurs (Fig. 2.3). The speed and magnitude of this response are easily visualized by recalling how quickly a splinter or similar injury causes inflammation and how much pus is produced.

Attachment

The initial contact of the PMN with an invading organism may be random. One of the most effective defenses bacteria have against phagocytosis is the capsule. This structure prevents attachment of the neutrophil's membrane to the organism, which must occur before ingestion can take place. Attachment is facilitated by the binding of specific antibodies or complement proteins to the microorganism. The neutrophil membrane has various receptors, including receptors for the Fc portion of IgG1 and IgG3, and the C3b component of complement. These three factors can bind to the invading

microorganism, resulting in the microorganisms being coated with one or more of these factors. The receptor on the PMN for the specific factor coating the bacterium binds to the factor and forms a bridge that brings the bacterium into close physical contact with the leukocyte membrane. The coating of the bacterium with antibody or complement components results in enhanced phagocytosis by the PMN. This process or phenomenon is called **opsonization**.

Ingestion and killing

The next step of phagocytosis is ingestion. This process occurs rapidly after attachment. The cell membrane of the phagocytic cell invaginates and surrounds the attached particle. The particle is taken into the cytoplasm and enclosed within a vacuole called a *phagosome* (Fig. 2.4). The phagocytosis of a particle triggers a significant increase in the metabolic activity of the neutrophil or macrophage. This increase is termed a *metabolic* or **respiratory burst**. The cell demonstrates increases in glycolysis, the hexose monophosphate shunt pathway, oxygen use, and production of lactic acid and hydrogen peroxide.

The phagosome is acidified by the formation of a proton (H^+) gradient. Highly toxic oxygen-reactive intermediates (e.g., hydrogen peroxide) and reactive nitrogen intermediates (e.g., nitric oxide) are secreted into the phagosome contributing to pathogen killing. Fusion with cytoplasmic granules (lysosomes), producing phagolysosomes, delivers a variety of antimicrobial factors. Lysosomes are vacuoles containing enzymes and other antibacterial components. The list of

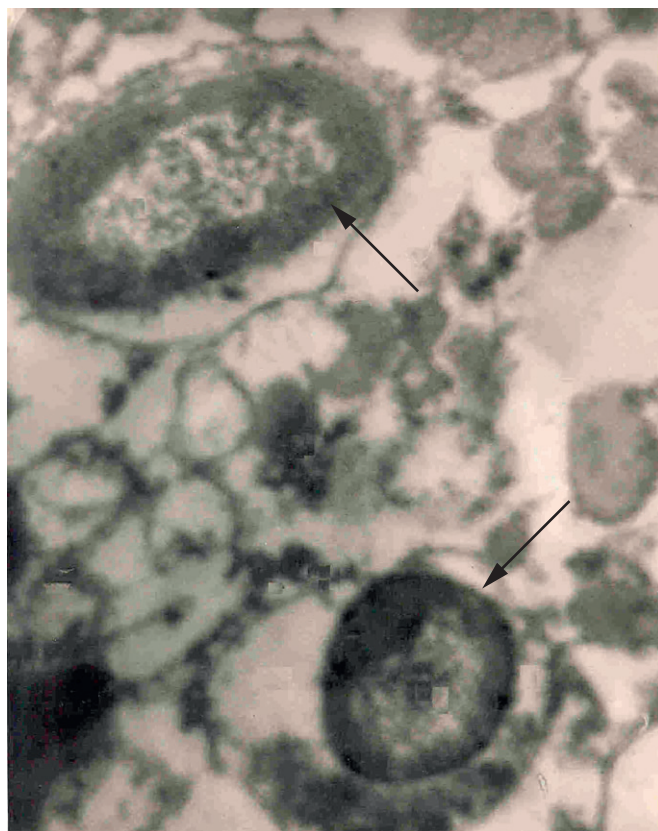


Fig. 2.4 Transmission electron micrograph of engulfed bacterial cells inside phagosomes (arrows).

enzymes found within the lysosomes is long—more than 20 enzymes, including proteases, lipases, RNase, DNase, and acid phosphatase. Other antimicrobial agents include lactoferrin, which chelates iron and prevents bacterial growth; lysozyme; and several basic proteins. The usual result is that a phagocytosed organism is quickly killed and digested. Neutrophils naturally contain many lysosomes and are effective at killing phagocytized pathogens. Alternatively, macrophages and monocytes require activation by factors such as interferon gamma and interaction with pattern recognition receptors, discussed later.

Organisms that are intracellular pathogens survive phagocytosis and can multiply within the phagocyte, most notably mononuclear leukocytes. Because PMNs have more lysosomes, intracellular pathogens are much less likely to survive in these cells. Other defense mechanisms must play a major role in immunity to these intracellular organisms. The importance of phagocytosis is seen in patients with defects in the numbers or function of phagocytic cells. Such patients have frequent infections despite possessing high levels of serum antibody. Many of the organisms listed in Table 2.4 are common isolates, which is not surprising because they have developed a means to interfere with phagocytosis, increasing their pathogenicity.

Inflammation

Inflammation is the body's nonspecific response to injury or foreign body. Fig. 2.5 illustrates the components involved in acute and chronic inflammatory responses. A hallmark of inflammation is the accumulation of large numbers of phagocytic cells. These leukocytes release mediators or cause other cell types to release mediators. The mediators cause erythema because of greater blood flow, edema from an increase in vascular permeability, and continued phagocyte accumulation via chemotaxins, resulting in pus. The enzymes released by the phagocytes digest the foreign particles, injured cells, and cell debris. After the removal of the invader, the injured tissue is repaired.

Immune responses

The immune system response to infection is briefly discussed in this chapter to provide the reader with an appreciation of its role and complexity. The balance between health and infectious disease is complex and mediated by humoral and cellular factors. The relative importance of each factor depends on the microbe, route of infection, condition and genetic makeup of the host, and other factors yet to be clearly characterized. For example, a patient with AIDS becomes more susceptible to opportunistic organisms as the immune system deteriorates.

Table 2.9 summarizes the defenses used by the human host against infection. Classically, the term *immunity* has been defined as a complex mechanism whereby the body is able to protect itself from invasion by disease-causing organisms. This mechanism, known as the **immune system**, consists of numerous cells and protein molecules that are responsible for recognizing and removing foreign substances. This general definition has been broadened over the years to mean a reaction to any foreign substance, including proteins and polysaccharides as well as invading microorganisms. Research advances over the years have dramatically increased our

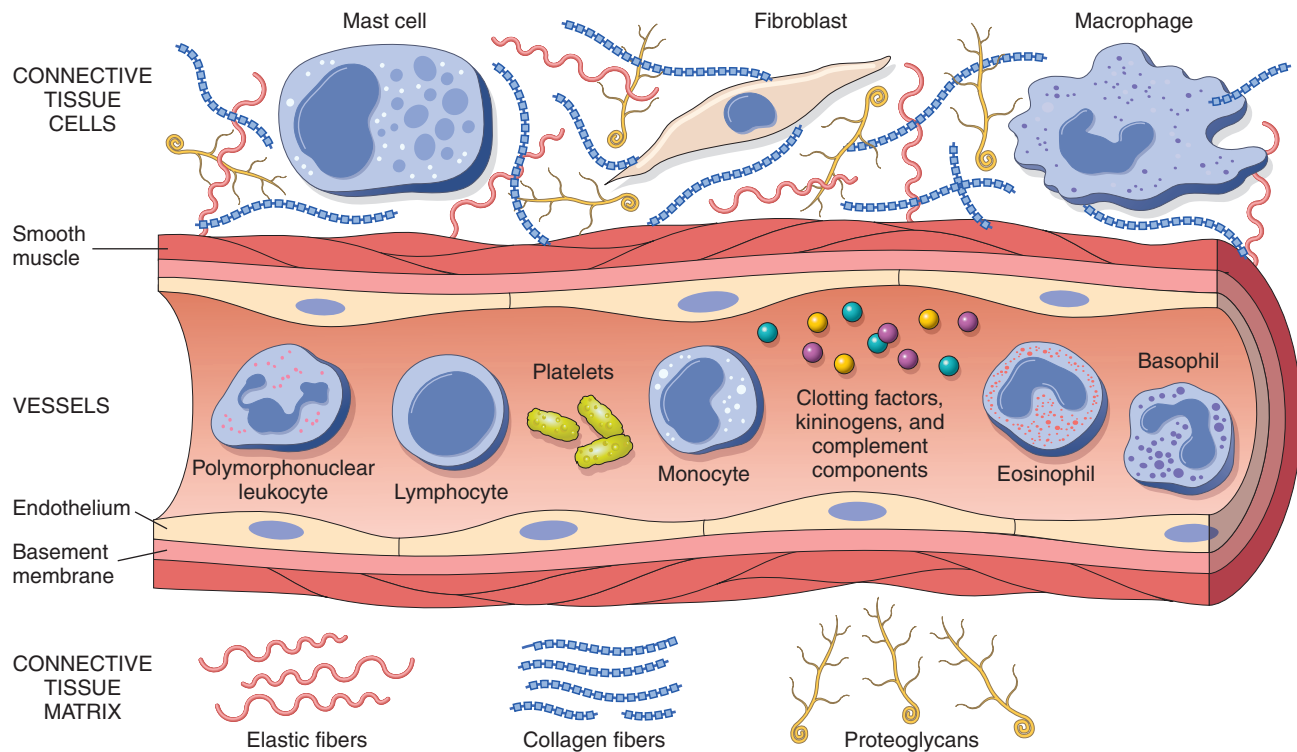


Fig. 2.5 Components involved in acute and chronic inflammatory responses. (From Kumar, V, et al. (2005). *Robbins and Cotran pathologic basis of disease* [7th ed.]. Philadelphia: Saunders.)

understanding of the **immune response** and given us an appreciation of the cellular interrelationships. The immune response can be divided into two broad categories: (1) *innate* or *natural immunity* with little or no specificity and (2) *adaptive* or *specific immunity*, which is highly specialized.

Innate, or natural, immunity

Innate immunity, also referred to as *natural* or *nonspecific immunity*, consists of several components. These include (1) physical and chemical barriers such as the skin and mucous membranes, (2) blood proteins that act as mediators of infection, and (3) a cellular mechanism capable of phagocytosis such as neutrophils and macrophages and other leukocytes such as natural killer cells. Fig. 2.6 shows examples of the innate immune defenses located at different body sites. This first line of defense has a limited capacity to distinguish one organism from another, and previous exposure to a particular foreign substance is not required. Physical barriers, mentioned earlier, may be as simple as the keratinized outer layer of the skin. Also, the secretions along the mucous membranes and the ciliated epithelial cells of the respiratory tract promote trapping and removal of microorganisms. In addition, many secretions provide a chemical barrier, such as the acidic pH of the stomach and vagina. Saliva and tears contain enzymes such as lysozyme, and the sebaceous glands of the skin contain oils and fatty acids capable of inhibiting invasion by pathogenic organisms. The normal biota of these sites adds another dimension to the host's ability to resist invading pathogens.

Once the physical and chemical barriers to infection have been penetrated, nonspecific mechanisms of innate immunity become involved—an animal's second line of defense. Innate

immune system cells have receptors called **pattern recognition receptors** (PRRs) that recognize conserved sequences on the surfaces of microorganisms. The first PRR discovered was the **Toll-like receptor** (TLR). These low-molecular-weight transmembrane proteins contain an extracellular domain that binds the pathogen-associated molecular patterns (PAMPs) that are present on pathogens. Eleven different TLRs (TLR1 to TLR11) have been identified in humans, and each has specificity for components of different microorganisms. For example, TLR4 recognizes LPS from many species of gram-negative bacteria. Recognition of these microbial components by PRRs allows innate immune system cells to initiate the innate immune response. PRRs on phagocytes can recognize PAMPs on pathogens and initiate phagocytosis.

Phagocytic cells ingest and kill microorganisms, while activated complement components contribute to a wide variety of immunologic events, including opsonization and chemotaxis, discussed previously. Collectively, these immunologic defense mechanisms, along with host tissue damage caused by the invading organisms, combine to produce an acute inflammatory response by the host. The importance of natural immune mechanisms rests in their rapid response to invading organisms. However, these mechanisms are effective primarily against extracellular bacterial pathogens, playing only a minor role by themselves in immunity to intracellular bacterial pathogens, viruses, and fungi.

Adaptive, or specific, immunity

Adaptive immunity enhances the protective capability of innate immunity. This arm of the immune response is specific for distinct molecules, responding in particular ways to

Table 2.9 Summary of defenses of the human or animal host to infection and evasion mechanisms attributed to various microorganisms

Host defense	Mechanism of evasion	Microbial example
Hydrodynamic flow	Attachment	Fimbriae, surface proteins, lipoteichoic acid
Mucous barrier	Attachment Penetration	Mannose-sensitive fimbriae, surface proteins Mucinase
Deprivation of essential nutrients (e.g., transferrin)	Systems of high-affinity uptake	Siderophores
Lysozyme in secretions	Resistance to lysis	Modification of peptidoglycan
Surface Igs	Absent or low immunogenicity Antigenic heterogeneity Masking of antigens Destruction of immunoglobulins	Hyaluronic acid, capsules Fimbriae, capsules, LPS, M protein Capsules, IgA-binding proteins IgA protease
Mucous membrane	Penetration	<i>Neisseria gonorrhoeae</i> , <i>Shigella</i> spp.
Serum defenses		
Recognition by antibody	Antigenic heterogeneity Masking of antigen Destruction of antibody Antigenic variation	Fimbriae, capsules, LPS, M protein Capsules, Ig-binding proteins <i>Borrelia</i>
Complement system	Failure to activate alternative pathway Inactivation of complement components Resistance to bacteriolysis Formation of abscess	Sialic acid capsules Cleavage of C3b CoIV plasmid <i>Bacteroides fragilis</i> capsule
Localization		
Fibrin tapping	Fibrinolysis	<i>Streptococcus</i>
Abscess formation	Collagenase, elastase	<i>Pseudomonas</i> , <i>Clostridium</i>
Adaptive immune response	Nonspecific B-cell activation Inhibition of cell-mediated immune response Rapidly fatal (toxin)	LPS, lipoprotein Anergy of miliary tuberculosis and T-cell destruction by HIV Anthrax, plague, <i>Clostridium</i>
Phagocytosis	Inhibition of chemotaxis Inhibition of attachment and ingestion Inhibition of metabolic burst Prevent phagosome-lysosome fusion Resistance to permeability-inducing cationic protein Resistance to oxidative attack Escape from phagosome Destruction of phagocyte	<i>Brucella</i> , <i>Salmonella</i> , <i>Neisseria</i> , <i>Staphylococcus</i> , <i>Pseudomonas</i> Capsules, M protein, Ig-binding proteins, gonococcal pili <i>Salmonella</i> Typhi Mycobacteria Gram-positive cell wall, smooth LPS, polyanionic capsules Catalase, superoxide dismutase, carotenoid pigments <i>Mycobacterium bovis</i> , <i>Legionella pneumophila</i> <i>Streptococcus pneumoniae</i> , <i>Streptococcus pyogenes</i> , <i>Staphylococcus aureus</i> , <i>Pseudomonas aeruginosa</i>

HIV, Human immunodeficiency virus; Ig, immunoglobulin; IgA, Immunoglobulin A; LPS, lipopolysaccharide.

different types of foreign substances and developing memory, which allows for a more vigorous response on repeated exposures to the same invader. Lymphocytes and their products, such as **antibodies**, are the major cellular mediators of the adaptive immune response. Antibodies are produced in response to **immunogens**, substances that can induce an adaptive immune response. An **antigen** is a molecule that can bind specifically to an antibody or T-cell receptor.

An interrelationship exists between the mechanisms of the innate and adaptive responses. For instance, inflammation, a nonspecific response of the innate system, provides a signal that triggers an adaptive immune response. In addition, the activity of **complement** (a component of innate immunity) to invading bacteria is enhanced by the presence of specific antibodies

(components of adaptive immunity). This activation leads to phagocytic clearance and elimination of the bacteria.

The adaptive immune response adds a high degree of specialization to the passive mechanisms of the innate response. The nature of the adaptive immune response varies according to the type of organism and is designed to eliminate it. For example, antibodies are produced by B cells and plasma cells in response to extracellular blood-borne organisms and aid in their elimination. However, the response to intracellular parasites is primarily by T cells that produce chemicals that enhance the activities of the phagocytic cells and a subset of T cells called *cytotoxic T cells*. The nature and origin of these cells are described later. Most importantly, the specific response remembers each time it encounters a particular foreign immunogen.

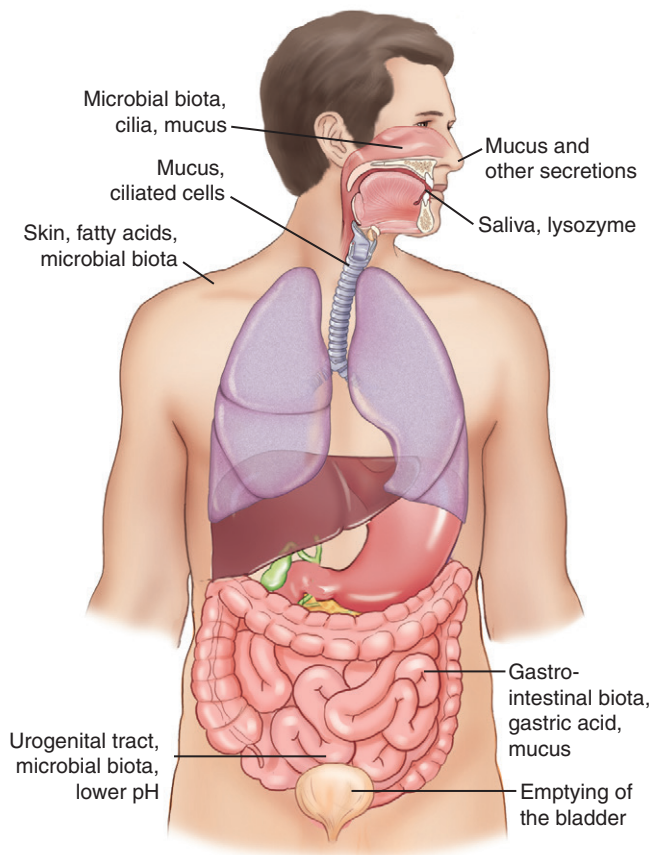


Fig. 2.6 Innate immune defenses located at different body sites.

This is called *immunologic memory*. Subsequent exposure to that immunogen stimulates an increased and specific defense. The adaptive immune response is the third line of defense and improves significantly the host's defense to infection.

Lymphocytes originate in the bone marrow from stem and progenitor cells. Lymphocytes mature and take up residence in various body tissues and organs, including the thymus, lymph nodes, and spleen. They are a diverse group of cells that can be classified into two major types based on cell surface markers: T (thymus-derived) cells and B (bone marrow-derived) cells. T cells mature in the thymus, while B cells leave the bone marrow as *transitioning B cells* and complete maturation in the spleen. The uniqueness of lymphocytes lies in the presence of specific cell surface receptor molecules that recognize and bind a unique immunogen, activating the cell to divide, differentiate, and secrete numerous effector substances. During embryogenesis, the millions of lymphocytes found in the body have been pre-engineered to recognize a vast array of substances as foreign while learning which substances constitute self. Thus the result of an encounter with the antigen is an expanded clone or clones of activated lymphocytes.

Nature of the immune response to infectious agents

Although both humoral-mediated immunity and cell-mediated immunity are important in protecting humans from a wide variety of infectious agents, each contributes differently according to the type of pathogen and virulence

mechanisms. While B cells play the predominant role in the **humoral immune response**, T cells mediate cellular immunity and play an important role in the humoral immune response. Antigen-presenting cells, like dendritic cells, process antigen and present it to T-helper cells with T-cell receptors specific for each antigen. Some T-helper cells become TH2 cells. Each B cell has surface receptors that recognize only one unique antigen. After antigen binding, the B cell processes the antigen, similar to the dendritic cell, and interacts with a TH2 cell with a T-cell receptor specific for the processed antigen the B cell is carrying. The TH2 cell secretes lymphokines that activate the B cell, triggering multiple cellular divisions and differentiation into *plasma cells* that actively secrete proteins called **immunoglobulins**, or antibodies. All antibody molecules derived from a single clone of B cells are of a single specificity (recognize a unique antigen), identical to the antibody receptor molecule on the original B cell. These antibody molecules circulate in the bloodstream and lymphatics, bathe tissues, and bind to infectious agents or substances to aid the host in eliminating them from the body. The detection and quantification of these antibody molecules, obtained from a patient's serum, constitute the primary goal of diagnosing infectious diseases through serologic methods.

Classification and characteristics of antibodies

Antibody molecules, found in serum and other body fluids and secretions, may be classified into one of five distinct immunoglobulin groups or classes. The classes differ from one another in several ways, including chemical structure, serum concentration, half-life, and activity.

The **immunoglobulin G (IgG)** antibody class constitutes about 70% to 75% of the total serum immunoglobulin pool. Their half-life in serum is 3 to 4 weeks. IgG can cross the placenta to the fetus, conferring some protection in both the pre-natal and the postnatal periods. Structurally, IgG is a protein with a molecular weight of about 150 kDa consisting of four polypeptides (two identical light chains and two identical heavy chains) bridged by several disulfide bonds (Fig. 2.7). Although the amino acid sequence of some regions of the polypeptides is nearly identical among all IgG molecules (conserved regions), one of the ends of each polypeptide is highly variable. These variable regions create two active fragment of antigen binding (Fab) sites on each IgG molecule (see Fig. 2.7). Thus IgG antibodies are said to be *bivalent*—capable of binding two antigen molecules.

Antibodies of the **immunoglobulin M (IgM)** class account for 10% to 15% of serum immunoglobulins. Their half-life in serum is about 5 days, and IgM cannot cross the placenta. A developing fetus in the second or third trimester as well as a newborn may respond to an infectious agent with an IgM antibody response of its own. IgM molecules are large; the molecule has a molecular weight of about 900 kDa and consists of a pentamer or five basic subunits—each composed of two heavy chains and two light chains (similar to an IgG molecule) and linked to another polypeptide chain (J chain) by disulfide bonds (Fig. 2.8). The IgM molecule has up to 10 antigen-binding sites available. IgM is the first antibody class produced in response to an immunogen. Both IgG and IgM antibodies are commonly assayed in a variety of serologic tests. The differences in size and configuration of IgG and

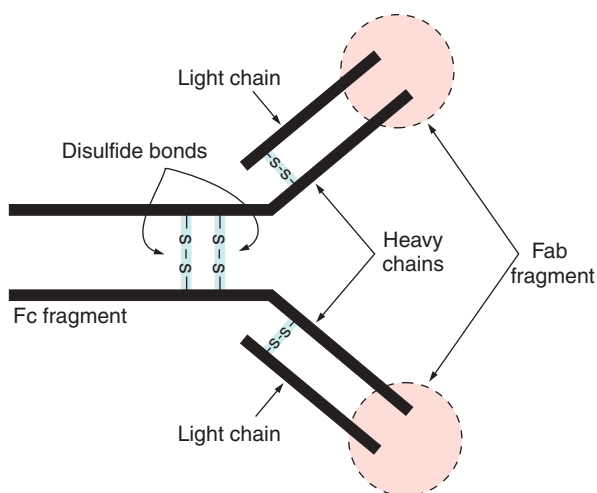


Fig. 2.7 Immunoglobulin G.

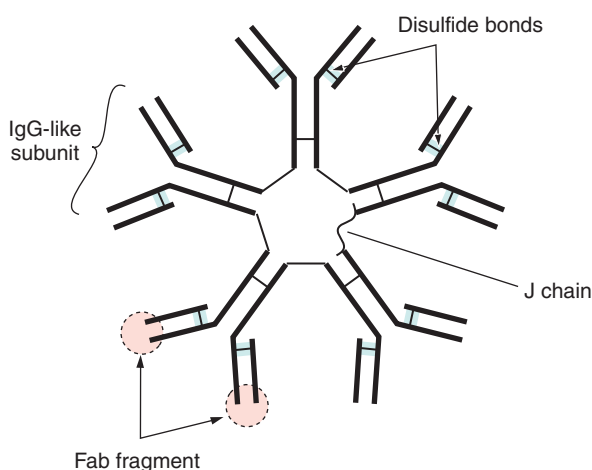


Fig. 2.8 Immunoglobulin M (IgM).

IgM molecules result in differences in functional activity of the molecules in serologic tests (Table 2.10).

Immunoglobulin A (IgA) antibodies represent 15% to 20% of the total serum immunoglobulin pool. IgA constitutes the predominant immunoglobulin class in certain body secretions, such as saliva, tears, and intestinal mucosa. Because of this association of IgA with mucosal surfaces, it provides protection against microorganisms invading at those sites. Serum IgA occurs primarily as a dimer composed of two subunits (each similar to an IgG molecule) linked together by a J chain. However, when found in secretions, the molecule also contains a secretory component that stabilizes the molecule. Although significant increases in serum IgA levels may occur in association with certain infections, the function of serum IgA is unclear, and few serologic tests for the diagnosis of infectious disease are designed specifically to detect IgA antibody.

The remaining two immunoglobulin classes, **immunoglobulin D (IgD)** and **immunoglobulin E (IgE)**, are normally found in very low concentrations in serum (<1%). IgE levels increase during infection by numerous parasites and may play a role in eliminating these infectious agents from the host. Total serum IgE levels might increase during parasitic infection, and IgE-specific serologic tests for the diagnosis of

Table 2.10 Immunoglobulin G versus immunoglobulin M

Property	Immunoglobulin class	
	IgG	IgM
Molecular weight	150 KDa	900 KDa
Number of 4-polypeptide subunits	1	5
Number of antigen-binding sites	2	10
Serum concentration (mg/dL)	800–1600	50–200
Percentage of total immunoglobulin in serum	75	10
Ability to cross placenta	+	–
Half-life (days)	23–25	5–8

IgG, Immunoglobulin G; IgM, immunoglobulin M; KDa, kilodalton.

a few parasitic agents have been developed. IgE also plays a role in some allergic (hypersensitivity) reactions, and elevated levels can be detected in these situations. The role of serum IgD during infection is unknown, except that it functions as a receptor on B lymphocytes for antigen.

Primary and secondary antibody responses

After exposure to an infectious agent, the host's acquired humoral immune response may produce various classes of antibody directed to one or more antigens associated with the agent. If the host has not been previously exposed to the antigen, a primary immune response that is characterized by the appearance of IgM antibodies occurs. IgM levels usually peak in 1 to 2 weeks followed by a gradual decline to undetectable levels over the next few months. At the time when IgM levels have nearly peaked, IgG (and in some cases IgA) antibodies become detectable, and their levels continue to increase for about 1 month, surpassing peak IgM levels. IgG levels remain elevated for months and then decline slowly, often persisting at low but detectable levels for years (Fig. 2.9).

A subsequent exposure to the same antigen elicits a secondary or **anamnestic immune response**, which is characterized by a rapid increase in IgG levels, prolonged elevation, and a more gradual decline (see Fig. 2.9). IgM synthesis plays a minor role in a secondary immune response. Serologic tests can distinguish between IgG and IgM antibodies. A positive test result for IgM is considered indicative of a current or very recent infection, whereas the presence of IgG alone suggests a previous infection or exposure. Similarly, the presence of significant levels of IgM (with or without IgG) in a newborn suggests in utero infection. IgM can be synthesized by the fetus but cannot cross the placenta, whereas IgG only in the newborn is indicative of passive maternal transfer of IgG across the placenta, not in utero infection.

Cell-mediated immune response

In contrast with humoral immunity, the primary effector cell in the **cell-mediated immune response** is the T cell. Like with a humoral immune response, antigen-presenting cells process antigen and present it to T-helper cells with T-cell receptors specific for each antigen. After interaction with the processed antigen, some T-helper cells become TH1 cells and undergo activation, differentiation, and cell division. The activated TH1 cell releases several low-molecular-weight

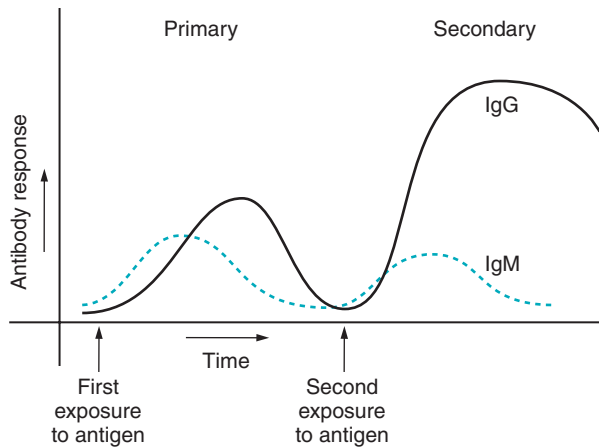


Fig. 2.9 Primary versus secondary response. *IgG*, Immunoglobulin G; *IgM*, immunoglobulin M.

proteins known as **lymphokines** that affect other cells, such as macrophages.

The cell-mediated immune response can also target host cells harboring intracellular pathogens, like viruses, for destruction. Cytotoxic T lymphocytes (CTLs) can recognize foreign antigens on the surface of infected cells. Through direct cell-to-cell contact, CTLs will lyse the target cell. Killing the host cell aborts the replication of the pathogen. Skin tests, such as the purified protein derivative for tuberculosis, can be used as a diagnostic tool to detect previous exposure to a pathogen. The in vitro measurement of cell-mediated immunity is beyond the scope of this book, and in vitro cellular immune function tests are not generally performed in microbiology or serology laboratories. Skin tests are used to detect cell-mediated immune response to some infections (e.g., tuberculosis).

In essence, the wide variety and complexity of infectious agents necessitate flexibility in the immune mechanisms of the host. Immunity to extracellular bacterial pathogens, such as *S. aureus* and *S. pyogenes*, is mediated primarily by antibody functioning either alone (neutralization of toxins and blocking the binding of bacteria to host cells) or with complement and neutrophils (chemotaxis and phagocytosis of bacterial cells). Immunity to intracellular bacterial pathogens, such as *M. tuberculosis*, is primarily cell mediated through the activities of T cells, lymphokines, and macrophages. If antibody is produced, it plays little role in eliminating this pathogen because the pathogen is sequestered (hidden) intracellularly, where antibody cannot reach.

Meanwhile, viral infections often elicit both humoral and cell-mediated immune responses. When viruses are free in the bloodstream or other body fluids, antibody can bind directly to adhesion molecules and neutralize viral particles by blocking attachment to host cells. For example, central nervous system infection by arboviruses (which cause encephalitis) or by certain enteroviruses (which cause meningitis) can be prevented if neutralizing antibody against these organisms is present when the virus reaches the bloodstream and before it enters the central nervous system. However, some viruses cause infections that spread cell-to-cell (e.g., herpes simplex virus); they would not be subject to the neutralizing effect of antibodies. In these cases, cell-mediated immunity plays a predominant role in eliminating the agent.

Immunity to both fungal and parasitic infections is also primarily cell mediated; antibody plays little or no role in

prevention of or recovery from infection resulting from these agents. Although antibodies may not have protective value for certain infectious agents, they nonetheless may have diagnostic and prognostic value in serologic tests. Methods for detecting the presence and significance of antibodies for diagnostic purposes are discussed in [Chapter 10](#).

Mechanisms by which microbes may overcome host adaptive defenses

Despite the host's elaborate defenses, infectious agents are obviously able to establish disease. The strategies that microbes employ to counter the host's defenses consist of inducing tolerance or immunosuppression, antigenic variation, and hiding inside host cells as intracellular parasites. Sometimes the host immune system fails to respond to specific immunogens of the infecting microorganisms. This failure to respond is not necessarily caused by immunosuppression. The inability to induce an immune response to a microbial antigen, referred to as *tolerance*, may be caused by a "weak antigen"—that is, an immunogen or immunogenic component of an organism that is incapable of stimulating an immune response by the host. This lack of response suggests tolerance to this immunogen. Tolerance to an infectious agent may also develop when the infection occurs during fetal life or in a neonate. For example, when infected by the rubella virus, a fetus makes its own antibodies. However, it is believed that the antibodies are often "weak" and unable to contain the infection. Because the T-cell response is also poor, the virus can persist in the fetus and during the neonatal period. Hence, infectious agents can persist if they are able to survive in the host during prenatal infections without producing an overt form of disease.

Certain infectious agents do cause immunosuppression in the infected individual. The decreased immune response is often more far-reaching than simply to the immunogens of the involved pathogen. Viruses, certain bacteria, and protozoans are examples of infectious agents likely to cause immunosuppression in the infected host. These agents multiply in macrophages or in lymphoid tissues. The exact mechanisms of immunosuppression have not been defined for all infecting agents. However, individuals infected with viruses such as Epstein-Barr virus and cytomegalovirus show depressed T-cell or antibody responses to other unassociated immunogens. Reduced immunoreactivity caused by an infectious agent is exemplified by that caused by HIV, which targets CD4⁺ T-helper cells. Because HIV destroys the major cells that defend the host against viral, fungal, and protozoan infections, the infected person becomes susceptible to opportunistic infections caused by these organisms.

Some microorganisms can change their surface antigens systematically during a single infection, while inside the host, evading the host immune defenses. This occurs in relapsing or recurring fever infections with *Borrelia recurrentis*. After an initial incubation period of 2 to 15 days following transmission of the spirochetes from a tick or louse, large numbers of the organism are found in the blood. The infected individual experiences high temperature, rigor, severe headache, muscle pain, and weakness. The febrile period lasts for about 3 to 7 days but ends quickly with the induction of an immune response. However, a similar but less severe course

of symptoms recurs several days to weeks later. The relapses are caused by antigen variation by the borreliae. The original antibody produced has low avidity (weak binding) to the new antigen. Spirochetemia worsens during the febrile period until a new antibody can be produced, and it diminishes between recurrences.

Intracellular parasites such as *Brucella* spp., *Listeria* spp., and mycobacteria avoid the host's immune response by surviving inside infected cells. This evasion strategy used by intracellular pathogens makes them unavailable as targets to the host's immune system. Macrophages that engulfed these microbial species protect them from antibacterial substances (e.g., antibody and complement) and support their growth inside the macrophage. During the exoerythrocytic cycle in liver cells, the malarial parasite *Plasmodium* spp. avoids being a target for the immune response. These parasites also infect red blood cells and cause disease while being protected from the host's defense mechanisms.

Hosts produce antibodies against specific immunogenic stimuli as part of an adaptive immune response. However, if the antibodies produced against an infecting organism are of low avidity or have a weak antimicrobial effect on the infecting organisms, the ability of the infected host to control the infection is decreased. Therefore in certain microbial infections, antibodies, although produced, provide little or no protection to the host.

POINTS TO REMEMBER

- Colonization of the body by microorganisms begins at birth.
- The resident microbial biota present at each site in the body are dictated by nutritional and environmental factors.
- Some species of the microbial biota may be opportunists that are capable of causing disease in an immunocompromised host.
- The microbial biota benefit the normal host by priming the immune system, outcompeting potential pathogens for nutrients, and creating a hostile environment for other microbes.
- True pathogens cause disease in all individuals a high percentage of the time.
- The host defense system against microorganisms includes physical, mechanical, and chemical barriers; components of the innate immune system, such as phagocytes, complement, and the products of inflammation; and components of the adaptive immune response.
- Microbes have mechanisms to evade the host's defenses, including the ability to evade phagocytosis; produce enzymes and exotoxins; induce tolerance in the adaptive immune system or suppress the adaptive immune system; and avoid recognition by the adaptive immune system by varying the antigens present on the surface of the microorganism.

LEARNING ASSESSMENT QUESTIONS

2. Which microorganism would most likely cause an opportunistic infection in the genitourinary tract of a woman of childbearing age?
 - a. *Candida albicans*
 - b. *Chlamydia trachomatis*
 - c. *Neisseria gonorrhoeae*
 - d. *Trichomonas vaginalis*
3. What caused the onset of diarrhea resulting from *Clostridioides difficile* infection in the patient in the Case in Point?
 - a. Stimulation of innate immunity producing intestinal inflammation
 - b. Decrease in the resident normal intestinal microbiota
 - c. Immune suppression from antimicrobial therapy
 - d. Side effect of antimicrobial therapy
4. Which organism(s) would be expected as contaminant(s) of improperly collected blood cultures?
 - a. *Bacillus* spp.
 - b. *Neisseria* spp.
 - c. *Streptococcus* sp. Viridans group
 - d. *Cutibacterium/Propionibacterium* spp.
5. A long-term resident species of bacteria in the gastrointestinal tract produces vitamin K, which is required for blood clotting in mammals. Which type of symbiotic relationship does this represent?
 - a. Commensalism
 - b. Mutualism
 - c. Parasitism
 - d. Sentinelism
6. An infant is born with IgM directed against cytomegalovirus (CMV) in the blood. What does this indicate?
 - a. An in utero CMV infection
 - b. A recent CMV infection in the mother
 - c. A CMV infection in the mother months before delivery
 - d. An outbreak of CMV infection in the delivery department
7. What is the most important benefit a capsule provides to a bacterium?
 - a. Enhances ability to infect host cells
 - b. Acts as a toxin
 - c. Degrades antibodies
 - d. Resists phagocytosis
8. What is the role of inflammation as a host immune defense?
 - a. Antibody production to neutralize invaders
 - b. T-cell activation and lymphokine production
 - c. Accumulation of phagocytic cells at the site of tissue injury
 - d. Cell-to-cell killing by cytotoxic T cells
9. Brucellosis is a disease occurring naturally in livestock like goats and cows. The causative agent can be transmitted to humans by drinking raw milk. Which of the following terms describes this disease?
 - a. Zoonosis
 - b. Vector
 - c. Carrier
 - d. Commensalism
10. Which part of an animal's defense mechanisms is most helpful in eliminating intracellular pathogens?
 - a. Phagocytosis by neutrophils
 - b. Cell-mediated immune response
 - c. Antibody production
 - d. Complement activation

11. What is the difference between resident and transient microbiota?
12. What is the significance of the carrier in the pathogenesis of disease?
13. What determines the composition of the indigenous biota at different body sites?
14. How does the resident microbial biota help protect the host from bacterial infection?
15. In the Case in Point, what virulence factor(s) did the infecting organism use?
16. What is the difference between true pathogens and opportunistic pathogens?
17. What is the difference between exotoxins and endotoxins?
18. What cells and soluble mediators are involved in the innate immune response versus the adaptive immune response? What cells and soluble mediators are involved in the humoral response versus the cellular response?
19. How does an animal's response to extracellular and intracellular parasites differ?
20. What are the steps involved in phagocytosis, and how can microorganisms evade each step?

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3

The laboratory role in infection control

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CHAPTER OUTLINE

General concepts in infection prevention and control practice, 51

Infection prevention and control in health care settings, 52

Infection prevention and control during a pandemic, 52

Infection control surveillance, 53

Frequently identified microbes, 55

Outbreak investigation, 56

Local outbreaks, 57

Widespread outbreaks, 57

Pandemic, 58

Steps of an outbreak investigation, 58

Investigation support from the laboratory, 59

Cultures and serology, 59

Environmental culturing, 60

Reporting, 61

Education, 62

Laboratory scientists and infection prevention and control practitioners, 62

Safety, 62

Response plans, 62

Bibliography, 63

OBJECTIVES

After reading and studying this chapter, you should be able to:

1. Delineate the various roles the laboratory and laboratory scientist play in an infection prevention and control program.
2. Describe the facilities and settings in which an infection prevention and control program is important.
3. Define surveillance and the ways in which surveillance is conducted.
4. Justify an outbreak investigation and the steps followed in an outbreak investigation.
5. Analyze the ways in which a microbiology laboratory can support an outbreak investigation.
6. Define when and how environmental culturing and surveillance cultures may be appropriate in an infection prevention and control program.

7. Identify microorganisms commonly encountered in healthcare-associated infections.
8. Describe agencies and entities to which an infection prevention and control program would provide reports.
9. Discuss educational activities that encompass an infection prevention and control program.
10. Correlate the activities of the laboratory with the safety and prevention activities of an infection prevention and control program.
11. Describe the roles of the microbiology laboratory and the epidemiology program in preparing for disease outbreaks, pandemic events, and potential bioterrorism activities.

KEY TERMS

Antibiogram

Antimicrobial pressure

Baseline data

Case definition

Catheter-associated urinary tract infection (CAUTI)

Central line-associated bloodstream infection (CLABSI)

Communal living

Community-acquired infection

Data mining

Emergency response plans

Emerging pathogens

Endotracheal

Environmental cultures

Epidemiologic curve

Extended care facility (ECF)

Hand hygiene

Healthcare-associated infection (HAI)

Iatrogenic

Index case

Infection control risk assessment

Infection prevention and control (IPC)

Infection preventionist (IP)

Infection rate

Intravascular device

Molecular epidemiology

Outbreak

Outbreak investigation

Pandemic

Prevalence

Public health

Pulsed-field gel electrophoresis (PFGE)

Reemerging pathogens

Standard precautions

Surgical site infection (SSI)

Surveillance

Surveillance cultures

Targeted surveillance

Total surveillance

Ventilator-associated pneumonia (VAP)

Case in point

An 82-year-old man was admitted to an intensive care unit (ICU) from an **extended care facility** (ECF); he was confused and short of breath. His chest radiograph revealed consolidation in the left lower lobe. A bronchoalveolar lavage specimen was sent to the laboratory for culture and antimicrobial susceptibility testing. An endotracheal tube was placed, and the patient was attached to a ventilator for respiratory support. The cultures grew a multidrug-resistant strain of *Acinetobacter baumannii*. Within 3 days, two more patients in the ICU had respiratory cultures positive for *A. baumannii*. Over the next 4 days, three additional ventilated patients had tracheal aspirate cultures that grew the same microbe.

The microbiology laboratory scientist notified the **infection prevention and control (IPC)** team of the unusual occurrence of several isolates of the resistant *Acinetobacter*. The IPC practitioner, microbiology laboratory scientist, ICU nurse manager, and physician in charge of the ICU met to review the situation. Additional patient testing was conducted to identify undetected carriers of the organism. Contact precautions were implemented for the patients. Education was initiated that highlighted hand hygiene practices, and environmental cultures were taken. The occurrence of the multidrug-resistant strain of *Acinetobacter* decreased, and eventually no new cases were observed.

Issues to consider

After reading the patient's case history, consider:

- The role of the microbiology laboratory in an IPC program
- The surveillance of healthcare-associated infections and the required laboratory support
- The information needed in an outbreak investigation
- The role of the laboratory scientist as an educator in IPC
- The need for prompt communication of results of concern to infection preventionists (IPs) in health care settings

Every year, an estimated 722,000 healthcare-associated infections (HAIs) occur that result in 75,000 deaths. Each day, approximately 1 in 31 US patients has at least one HAI. HAIs are infections that originate in health care facilities. These infections cost the health care system countless dollars. An effective infection control program must be established in a health care setting to recognize and prevent HAIs.

With the advent of terrorist activities worldwide, the microbiology laboratory has become an integral part of that area of the infection control program. Whether dealing with emerging diseases, such as the Zika virus in 2016, the SARS-CoV-2 (severe acute respiratory syndrome–coronavirus-2) pandemic in 2019, or reemerging diseases, such as Rocky Mountain spotted fever, the laboratory must stay closely aligned with the infection control activities in the setting that the laboratory serves. **Box 3.1** lists examples of **emerging pathogens** and **reemerging pathogens**.

Infectious agents that have not been previously recognized sometimes appear. Several agents have emerged during the past two decades that include the West Nile virus; the avian influenza virus; the SARS-CoV virus, which is responsible for outbreaks of severe acute respiratory syndrome (SARS); the MERS-CoV virus, which causes Middle East respiratory syndrome (MERS); and most recently, in 2019, the SARS CoV-2

BOX 3.1 Examples of emerging and reemerging pathogens

Emerging pathogens

- Avian influenza virus
- Coronavirus (SARS and SARS-CoV-2)
- Hemorrhagic fever viruses
- West Nile virus
- Zika virus

Reemerging pathogens

- Anthrax (*Bacillus anthracis*)
- Botulism (*Clostridium botulinum*)
- Plague (*Yersinia pestis*)
- Rocky Mountain spotted fever (*Rickettsia rickettsii*)
- Tularemia (*Francisella tularensis*)

virus, the coronavirus that causes COVID-19 (coronavirus disease 2019).

The laboratory, therefore, must quickly learn not only how to rapidly identify these agents in human infections but also how to collaborate with the IP in dealing with these agents in the human population. Questions that the IP must ask include the following:

- How are these infectious agents spread or transmitted?
- What reservoirs can harbor them?
- What is their incubation period?
- What antimicrobial agents can successfully treat them?
- How can the environment be disinfected?

The laboratory scientist, IP, and infectious disease physicians all must combine their knowledge to address these issues. The educational role of the laboratory scientists become evident in teaching specimen collection, specimen transport, disinfection, and safety methods.

The reemergence or potential reemergence of pathogens once thought to be eliminated demands collaboration with the IP. The pathogens that cause Rocky Mountain spotted fever, anthrax, and plague are agents that clinical microbiologists have learned about but, because these diseases are uncommon, are not often identified by microbiology laboratories. The laboratory scientist must relearn information once thought to be of low importance. Media selection, identification techniques, and safety precautions must all be reexamined and implemented. Interaction with the IP strengthens the establishment of prevention and control strategies. This chapter covers the settings, activities, and analytic practices involved in implementing an effective infection control program, specifically from the laboratory scientist's perspective.

General concepts in infection prevention and control practice

The clinical laboratory and laboratory scientists play a vital role in the functioning of an effective infection prevention and control (IPC) program. In some settings, the laboratory scientists provide supportive data; in other settings, they may function as a part-time **infection preventionist** (an **infection control practitioner**) as well as a part-time laboratorian. Not

infrequently, the laboratory scientist may leave the laboratory and become the IP or work as a member of an IPC department. There are multiple pathways to join the discipline of infection prevention, including continuing education, specialized training in infection prevention, and pursuing career opportunities.

As the term implies, *infection prevention and control* involves activities aimed at preventing and reducing the dissemination of infections in persons in broad and varied settings. The field is highly interdisciplinary by definition and incorporates many departments and services in health care facilities. The activities range from surveillance activities to outbreak investigation to data management to education. They require an inquisitive and analytic mind, both of which are characteristics seen in clinical laboratory scientists.

Infection prevention and control in health care settings

IPC practices are important in many health care settings. Table 3.1 lists different health care environments in which infection control practices play a vital role. In all the settings, the goal is to prevent the spread of infectious agents and reduce dissemination of infections by assisting with assessment, planning, implementation, and evaluation of national infection control practices. In **public health** or community settings, the transmission of microorganisms occurs through many events in daily living. Microbes are spread at home, in daycare centers, in schools, in crowds, and by individuals. There are sexually transmitted diseases (STDs), diseases spread by respiratory routes, diseases spread by direct contact, foodborne diseases, and waterborne diseases, all of which occur in the public arena.

Infections occur as community-acquired infections, HAIs, and iatrogenic infections (infections caused by activities of a health care provider such as a physician or therapist). HAIs and iatrogenic infections occur because of instrumentation, increased use of antimicrobial agents, breaks in aseptic techniques, and lack of hand hygiene. In acute care hospitals, HAIs present as surgical site infections (SSIs), central line-associated bloodstream infections (CLABSI), catheter-associated urinary tract infections (CAUTIs), and

ventilator-associated pneumonia (VAP), among others. Over 22,000 SSIs and 62,000 device-associated infections were reported by US hospitals in the 2019 HAI progress report. They are found in adults, teenagers, children, neonates, healthy individuals, and immunocompromised patients.

Ambulatory care settings include outpatient surgery, chronic dialysis, and infusion centers; physician or therapeutic practices; and emergency care facilities (ECFs). Infection control must be practiced in these settings, even though the patients may not be seen again. In ECFs, patients are frequently immunosuppressed by disease, age, or therapy. ECFs include skilled nursing facilities, nursing homes, assisted living centers, rehabilitation centers, and hospice care settings. Because of their suppressed defenses and frequent contact with others, these patients are prime candidates for acquiring infections.

Patients who are at home and receive home care from family or a professional home care provider sometimes acquire infections. Most often, home care involves intravascular-related or device-related care. As in ECFs, the immune defenses of these patients are often suppressed by their disease or therapy.

Communal living programs can also be settings in which infections occur. These community living programs include prisons and behavioral health facilities. In these facilities, infections might be found that are spread by contact (illicit tattooing), by intimate contact with blood and body fluids, or simply by aerosols and droplets from overcrowding.

The microbiology laboratory may receive specimens from any of these settings, all of which represent opportunities for infections to spread and opportunities for infection control to be practiced. Microbiology laboratory scientists must know who their clients are and be poised to insert themselves into infection control activities for the varied customer base.

Infection prevention and control during a pandemic

Because of the high transmissibility of SARS-CoV-2, the Centers for Disease Control and Prevention (CDC) recommends using additional IPC practices during the COVID-19 pandemic, along with standard practices recommended as a part of routine health care delivery to all patients. These practices are intended to apply to all patients, not just those with suspected or confirmed SARS-CoV-2 infection.

In assisted living facilities, nursing homes, and long-term care facilities, given the amass population and close proximity, the risk for spread of SARS-CoV-2 among their residents is high. Residents in these health care settings are often older adults with underlying medical conditions and therefore are at increased risk for severe illness if infected with respiratory and other pathogens. Therefore the CDC recommends source control measures for all persons, including when in a health care setting, and an effective IPC program that is critical in protecting both residents and health care personnel. Recommendations that are specific to these facilities are provided by the CDC that complement but do not replace the general CDC IPC recommendations for SARS-CoV-2 for health care personnel during the COVID-19 pandemic. The complete guidance and recommendations may

Table 3.1 Health care settings involving infection prevention and control

Setting	Description
Public health	Community settings (e.g., daycare centers, schools)
Acute care	Hospitals
Ambulatory care	Outpatient surgery, chronic dialysis and infusion centers, physicians' offices, emergency and urgent care facilities
Extended care	Skilled nursing facilities, nursing homes, assisted living centers
Home care	Skilled nursing provided in the home
Communal living	Prisons and jails, behavioral health facilities, residential schools

be found at <https://www.cdc.gov/coronavirus/2019-ncov/hcp/long-term-care.html>. Key points in the guidance include the following:

- Visitation and physical distancing measures
- ICP guidance for health care professionals on the proper use and handling of personal protective equipment (PPE)
- Guidance about when it may be appropriate for a resident suspected of SARS-CoV-2 infection to “shelter-in-place”
- Quarantine recommendations and management of residents who had close contact with someone with SARS-CoV-2 infection

Infection control surveillance

IPC programs include many activities aimed at preventing the spread of infections. To determine where to most efficiently direct these preventive activities, an effective IPC program must collect data on existing infections. By comparing baseline figures with periodic numbers, the IP can recognize an **outbreak** (sudden increase in the occurrence of a disease), upward trends, and positive effects of interventions. Ongoing, systematic collection of these data and the analysis and interpretation of the details surrounding a disease or event is termed **surveillance**. The laboratory contributes to these records on a daily basis. Prompt and efficient communication with the IP and public health authorities is a critical function of the laboratory’s role in surveillance.

During the COVID-19 pandemic, the World Health Organization (WHO) published surveillance strategies to prevent the spread of the disease. While rigorous public health and social measures were implemented to slow the spread of COVID-19, it is critical that an effectual surveillance is in place to guide ongoing implementation of control measures. The key considerations for and objectives of surveillance include the ability to perform rapid detection, isolation, manage suspected cases, and guide the implementation of control measures. Surveillance programs should also be able to detect and contain outbreaks among vulnerable populations and include mandatory and immediate reporting of notifiable diseases. Lastly, the WHO recommends that surveillance programs assess how the pandemic affects health care systems and society as a whole.

Surveillance definitions

An important part of IPC is surveillance; data can be compared internally, locally, and nationally if standard definitions are used. [Table 3.2](#) lists the common terms used in surveillance and their definitions. The CDC recommends definitions that are generally used in the infection control profession. Most IPs are concerned about infections that are acquired within the health care facility, i.e., HAIs. These infections occur after the patient arrives (generally not within the first 48 hours) and were not incubating in the community before the patient arrived. HAIs can be followed within a setting to determine when infection numbers increase above baseline, thereby requiring investigation and intervention.

Infections are generally defined by site, risk factors, and procedures, as shown in [Table 3.2](#); primary infections are infections that occur at one site (e.g., a primary bacteremia). A primary bacteremia would not have another site as the source

Table 3.2 Surveillance definitions

Term	Definition
Primary infection	Infection related to a single, specific site, not from multiple sites
Bloodstream infection	Infection found in the bloodstream
Central line–associated bloodstream infection (CLABSI)	Bloodstream infection related to the presence of intravascular devices such as a central venous catheter or a peripherally inserted central line (PICC)
Surgical site infection (SSI)	Infection at a site where a surgical procedure was performed; usually risk is stratified by length of surgery, site of infection, degree of anticipated contamination, and some host risk factors
Urinary tract infection	Infection of the urinary tract, frequently associated with a urinary catheter
Ventilator-associated pneumonia (VAP)	Pneumonia in a patient associated with mechanical ventilation

of the infection, as would be seen in urosepsis, in which case the primary site would be the urinary tract. Using primary bacteremia as an example, the laboratorian recognizes a bloodstream infection (BSI) by the recovery of clinically significant organisms from blood cultures, whereas the IP determines whether the infection was healthcare-associated and primary. If the IPC was monitoring CLABSIs, the IP would determine whether the BSI was related to an **intravascular device**. For this reason, the site of the blood culture draw (e.g., peripheral internal jugular catheter, femoral catheter) is important to include with the specimen description on the microbiology laboratory report.

The incidence of SSIs is frequently reviewed by the IP. To the laboratorian, the isolation of pathogens from the surgical site is indicative of postsurgical wound infection; the IP determines whether the infection is superficial or deep or if it was an organ space infection. Therefore the site of the wound culture is an important component of the specimen description on the laboratory report. The IP relates the infection to other risk factors, such as the length of surgery, degree of contamination of the surgical site (gunshot wound to the abdomen vs. hernia repair), and whether any breaks in surgical technique occurred.

Because urinary tract infections (UTIs) are determined by microbial growth from a urine specimen, the method of urine specimen collection should be described and differentiated between a voided clean-catch urine specimen and a specimen collected by catheterization. The definition of a healthcare-associated UTI includes the presence or absence of a urinary catheter. Healthcare-associated pneumonias are difficult to assess from the perspectives of the laboratory and IP. From the IP perspective, the criteria to define a hospital-acquired pneumonia include the presence of an **endotracheal** tube or some other respiratory device and whether the pneumonia was incubating or present when the patient arrived. In the laboratory, the microbiologist should include in the specimen description on the laboratory report the type and source of the respiratory specimens (e.g., bronchoalveolar lavage, tracheal aspirate, expectorated sputum). With sputum specimens, the quality of the specimen, as evaluated by the presence of white

blood cells and single bacterial morphotypes and the absence of squamous epithelial cells in the direct smear, is an important adjunct. The use of these definitions is important for guiding the IP and allowing the comparison of data.

General or targeted surveillance

IPC programs may have all HAIs within the setting under surveillance, or they may be observing for only specific infections because of budget or personnel constraints. In **total surveillance** programs, all infections are recorded and analyzed to determine whether the infections are healthcare-associated. Risk assessments determine whether the situation is high or low risk and whether the infections are occurring in an unanticipated number. The **infection rate**, which is the speed of spread or frequency of an infectious disease within a population, is determined and analyzed to establish whether the number of infections has increased or decreased. These rates can be compared with previous rates in the setting or with rates in similar local or national health care facilities.

There are several ways of calculating infection rates. For example, the SSI rate is defined as the number of infections per number of procedures expressed as a percentage. Infection rates of CLABSI and VAP are calculated as the number of infections per 1000 device days. If an unexpected change in rates is seen, the IP may conduct further investigations to determine, if possible, the cause of the change and to propose a course of action to reverse the situation. Testing of selected patients or environmental sources, called *surveillance cultures*, may be necessary to clarify the source of organisms being transmitted. From the results of the investigation, the IP would recommend appropriate interventions, such as a change in procedure, education, or increased emphasis on hand hygiene. The IP would continue to monitor the infection to determine whether the infection rate decreases.

Unlike in total surveillance, **targeted surveillance** involves a close watch of only specific, high-risk, high-volume procedures. For example, one program may follow only CLABSI in the ICU and SSIs in gynecologic surgical procedures, whereas another program may conduct surveillance on VAP in the neonatal ICU and adult ICU. Which infections to target is largely based on review of previously ascertained data on infection rates (**baseline data**), on recognition of high-volume and high-risk procedures within the setting, and sometimes on requests by others (e.g., insurance companies, physicians). This process is often termed a *risk assessment*. The targeted surveillance may change from year to year, depending on recognized outbreaks or changes in the number of procedures performed. All surveillance data must be carefully collected, use high-quality laboratory data, and be protected from legal discovery, as determined by the risk management team for the setting.

Baseline data

To recognize when infections constitute an outbreak or when an upward trend is occurring, the IPC program must have established baseline data, that is, the historic occurrence of infections over time. Data at the national level derived from the National Healthcare Safety Network (NHSN) can also serve as the baseline data. However, baseline data from within the specific health care setting more accurately reflect events within that setting. The baseline data are collected and analyzed by numbers, by percentages, or by rates per 1000 device days. Baseline data are an important component of the decision making process

regarding which types of infections to target. In 2016, ambitious targets were set for reduction of specific HAI rates between 25% and 50%. These targets were not met by 2020, so much work remains to be done in this area of health care.

Data gathering

Culture review

Many infection control programs rely heavily on a review of culture results, which is initiated informally by the laboratory scientist, who may then inform the IP of what appears to be an increased number of specific isolates. For example, the laboratory scientist may notice the isolation of an increased number of *Serratia marcescens* or *Salmonella* spp. isolates, which may be infrequently seen in this particular health care facility. Or the laboratory scientist might think that there is an unusual number of methicillin-resistant *Staphylococcus aureus* (MRSA) isolates from skin sites in a prison setting. The appearance of an increase in the number of surgical site specimens with various microorganisms may warrant contacting the IP. These are informal data-gathering activities that are significant to the IPC program. To report data, laboratorians use their experience and sense of what is usual regarding types of specimens and microbes encountered.

A more formal data analysis involves the daily review of culture results reported by the laboratory. Such a review requires laboratory-generated results, generally from a laboratory information system (LIS). For example, the IP or microbiologist reviews positive cultures and categorizes them into groups based on sites, units, organisms, or procedures. From this categorization, the IP may recognize a trend, such as an upward trend in the number of *Pseudomonas aeruginosa* isolates from a skilled nursing facility or an increased number of positive cultures in laminectomy patients. Even more important is when the IP determines that there are more *S. aureus* isolates than usual in the home health care setting. In this case, an initiation of detailed investigations may be indicated. These fact-gathering activities are more formal and specific than the informal monitoring done by the laboratory scientist on the basis of experience.

It is critical to establish a relationship between the laboratory and IP, especially when the laboratory is located off-site from the health care setting. This will facilitate communication of concerns identified by the laboratory professional or when changes in laboratory procedures are considered or made. Collaboration is key to effective outbreak control. Diligence is also needed to collaborate on performance improvement projects to reduce infection risks to patients, staff, and visitors.

Case check 3.1

In the Case in Point, a multidrug-resistant strain of *A. baumannii* was recovered from the bronchoalveolar lavage of the index case. Within days, respiratory cultures from a number of patients grew *A. baumannii*. This upward trend was reported to the IP, and intervention measures were administered.

Statistical correlation by interfacing with the LIS offers a more sophisticated data review system for the purpose of **data mining**. In this type of data analysis, a multitude of events

from a database are searched, analyzed, and then reported to the IP. Data mining removes much of the daily details of the culture result review. It also adds other health care parameters to the analysis that are frequently not available to the IP without detailed examination. This type of analysis may require the LIS and other hospital information systems to interface with the data mining system.

No matter how the culture results are screened and analyzed, a timely review is an integral component of an infection control program. Not only are the patients and their health care providers dependent on high-quality microbiology results, but the effectiveness of an infection control program is also dependent on these results.

Cases

The laboratory must be attuned to what is happening in the community and in various community settings in regard to infection control issues. Infection control in the public health arena affects the local microbiology laboratory. For example, there may be an increase in the number of cases of pertussis in surrounding counties. Even though the laboratory in the immediate county may not have seen any *Bordetella pertussis* isolates, it must be aware of these other cases. Similarly, the anticipated appearance of influenza cases is a seasonal occurrence that also influences the activities of a microbiology laboratory. The laboratory can be proactive in educating health care providers on specimen collection and transport if those are unique to a specific public health concern. Awareness of infection control activities within the public health setting allows the laboratory to acquire the necessary media or reagents to meet emerging needs.

Potential infection control issues that might become apparent within a setting can drive activities in the microbiology laboratory. For example, a product used in the health care setting is recalled because of suspected bacterial contamination. Effective communication between the infection control program and microbiology laboratory is needed to establish screening techniques in anticipation of cases involving contaminated products that might arise in the health care setting. Laboratory scientists must increase their knowledge of the specific product, types of specimens to anticipate, and expected isolates, which may be unique or previously unrecognized by the laboratory. Collaboration among the microbiology laboratory, IPC program, and health care customer becomes paramount. Reporting of community-acquired cases to the laboratory and by the laboratory to the IPC team is an important step in surveillance activities.

Laboratory support and data gathering

Besides providing culture results, the microbiology laboratory also provides the IP with other details. These may include specimen contamination rates, numbers of isolates per site, and/or number of isolates per unit within the health care facility. Knowledge of specimen contamination rates helps the IP with the interpretation of culture results and provides guidance in developing educational activities to improve the collection of quality specimens. For example, if respiratory specimens are frequently contaminated with upper respiratory tract microbiota, interpretation of the results for the health care provider and IP becomes more difficult. Contaminated specimens also prove costly for the patient and health insurance payer because they do not provide useful or appropriate information, and cultures might

need to be repeated. Attempts to determine VAP become futile if the specimens are contaminated with upper respiratory tract microbiota. A similar situation is encountered in blood culture contamination, which lessens the likelihood of high-level interpretive abilities. Blood culture contamination rates above 3% are generally considered high and may indicate educational opportunities for the microbiology laboratory and infection control program. However, it is generally difficult to lower the contamination rate below 2%.

The types of pathogens isolated from given specimens represent important information that can be generated by the laboratory to support the IPC program. For example, if *S. aureus* is frequently isolated from skin infections in a jail setting, the health care provider can anticipate the success of antistaphylococcal antimicrobials in routinely treating those infections. If MRSA, however, is more frequently isolated, the health care provider would change the empiric therapy.

The **prevalence** of a particular pathogen is another piece of information that the microbiology laboratory can provide to the IP. Prevalence is the number of cases of a disease that occur in a given moment in time or specific period in a given population. Therefore not only knowing what pathogens are isolated from a given body site but also being familiar with what pathogens are frequently isolated from a given location in a health care facility is important to the IP. For example, a skilled nursing facility may be reassured that the lack of negative air flow rooms is acceptable if *Mycobacterium tuberculosis* has not been isolated from any of its patients in the past 7 years.

Being able to recognize which pathogens are isolated from patients in an ICU may provide the opportunity for the IP to inform health care providers about the effects of **antimicrobial pressure**. For example, if extended-spectrum β -lactamase (ESBL)-producing *Klebsiella pneumoniae* isolates were seen in that ICU, the physicians might be advised to limit the use of antimicrobial agents that tend to induce the formation of ESBLs. The microbiology laboratory then serves as an important adjunct to an infection control program by providing a multiplicity of types of data.

Frequently identified microbes

Although the types of microorganisms seen in HAIs vary among settings, common pathogens are encountered. This discussion of the laboratory's role in IPC addresses some of the common microbes the laboratory scientist will encounter in the health care setting and the typical body sites they infect. [Table 3.3](#) lists some of the most common organisms identified. The most frequent HAIs are pneumonia, SSIs, gastrointestinal illness, UTIs, and primary BSIs.

Public health and community setting

The laboratory serving a public health or community setting is likely to identify infectious diseases of infection control importance that are less frequently seen in other health care situations. Some of the microbes frequently associated in waterborne or foodborne community outbreaks include *Giardia lamblia*, *Cryptosporidium*, *Salmonella*, *Shigella*, and *Campylobacter*. STDs such as chlamydia, gonorrhea, syphilis, and acquired immunodeficiency syndrome are community-acquired infectious diseases identified in public health laboratories and laboratories serving acute care facilities. Some

Table 3.3 Health care settings and common agents of infection control significance

Health care setting	Microorganisms and infectious diseases
Public health	<i>Neisseria meningitidis</i> , <i>Salmonella</i> spp., <i>Shigella</i> spp., <i>Campylobacter</i> spp., <i>Giardia lamblia</i> , <i>Cryptosporidium</i> spp., <i>Chlamydia</i> spp., <i>Treponema pallidum</i> subsp. <i>pallidum</i> , <i>Neisseria gonorrhoeae</i> , HIV, hepatitis B virus, hepatitis C virus
Acute care	<i>Clostridioides difficile</i> , <i>Staphylococcus aureus</i> , MRSA, <i>Enterococcus</i> spp., <i>Escherichia coli</i> , <i>Klebsiella pneumoniae</i> , <i>Pseudomonas aeruginosa</i> , <i>Acinetobacter baumannii-calcoaceticus</i> , coagulase-negative staphylococci, <i>Candida auris</i>
Ambulatory care	Hepatitis B virus, hepatitis C virus, HIV, <i>S. aureus</i> , MRSA, <i>P. aeruginosa</i> , <i>Enterococcus</i> spp.
Extended care facilities, home care	<i>C. difficile</i> , <i>P. aeruginosa</i> , <i>S. aureus</i> , MRSA, <i>Acinetobacter</i> , <i>Enterococcus</i> spp., coagulase-negative staphylococci, <i>C. auris</i> , <i>Candida albicans</i> , scabies
Communal living	<i>S. aureus</i> , MRSA, hepatitis C, <i>Pediculosis</i> spp. (lice), <i>Sarcoptes scabiei</i> (scabies)

HIV, Human immunodeficiency virus; MRSA, methicillin-resistant *Staphylococcus aureus*.

organisms that are important in public health settings may be more likely to be recovered in an acute care hospital but are reported to a public health jurisdiction for the latter to follow up on as a potential outbreak. Examples include agents such as *Neisseria meningitidis*, encephalitis viruses, West Nile virus, and coronaviruses SARS and COVID-19.

Recently, an emphasis has been placed on agents of bioterror. This is the use of infectious agents or microbial and plant toxins to instill terror in a population (see [Chapter 30](#)). Likely agents are those that are uncommonly seen in clinical laboratories, have a significant mortality rate, and can be difficult to diagnose, treat, and control. Examples include *Bacillus anthracis*, *Brucella* spp., *Burkholderia mallei*, *Francisella tularensis*, *Yersinia pestis*, and botulinum toxin, among others.

Acute care setting

Although a great variety of infectious agents can cause concern as HAIs in acute care settings, some are more frequently seen than others. These are listed in [Table 3.3](#). *S. aureus*, especially MRSA, is an important healthcare-associated pathogen that causes BSIs, SSIs, VAP, and other infections. Although community-acquired MRSA has become more prevalent, healthcare-associated MRSA is worrisome because of its resistance to multiple antimicrobial agents and the opportunity to infect a compromised population of patients. Similarly, enterococci are pathogens of concern in HAIs because of their possible resistance to vancomycin and their potential ability to pass this resistance on to other bacteria. Antimicrobial resistance is considered a major threat to human health. Globally, an estimated 1.27 million deaths directly attributable to microbial resistance occurred in 2019. The leading causes of these infections were *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella*

pneumoniae, *Streptococcus pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*.

Escherichia coli and other members of the order Enterobacterales, such as *K. pneumoniae*, are common fecal organisms seen in a variety of HAIs in various sites, including BSIs, SSIs, VAP, and UTIs. In patients who are immunosuppressed by disease or therapy, *P. aeruginosa* is seen as a cause of HAIs. *Clostridioides difficile* is an organism whose toxins cause diarrhea in healthcare-associated infections. Recovery of the organism is not the significant finding, but demonstration of the toxin or detection using nucleic acid amplification testing in a symptomatic patient's stool is significant.

Ambulatory care setting

Ambulatory care settings include a variety of different locations. However, the commonly encountered microorganisms of infection control importance do not vary. Many patients have a chronic illness, are immunosuppressed, and have infectious diseases caused by opportunistic pathogens. Other patients are likely to acquire community-acquired infections, such as hepatitis B virus, hepatitis C virus, and human immunodeficiency virus (HIV) infection. Bacterial isolates include pathogens seen in other settings, such as *S. aureus*, MRSA, vancomycin-resistant enterococci (VRE), and *P. aeruginosa*.

Extended care facility and home care settings

Patients in ECFs and home care settings are frequently immunosuppressed by disease or therapy and often need intravascular or other device-related care. The microbes identified in these patients are often opportunistic pathogens. Causative agents of infection control significance identified in these patients include *P. aeruginosa*, *Candida* spp., *S. aureus*, MRSA, VRE, *Acinetobacter* spp., *C. difficile*, and multiple-antifungal-drug-resistant *Candida auris*. The risk for SARS-CoV-2 spreading among these residents is also high.

Communal living

People who are housed together in some form of communal living setting, such as a prison or behavioral health facility, often have common pathogens similar to those in the other settings described earlier. The infectious diseases are more likely related to the activities of the persons in the facility. For example, *S. aureus* and MRSA are recovered from prisoners who practice illicit tattooing with nonsterile shared equipment, whereas lice, scabies, and hepatitis C virus are more frequently seen in behavioral health settings because of the community source of the clients and their intimate contact with skin and belongings, or with blood and body fluids, respectively.

Outbreak investigation

When a sudden increased frequency of a disease above the usual rate or what is expected in a given population or geographic area occurs over a period, an *epidemic* may be suspected. Most cases are presumed to have a common cause or source or to be related to each other in some way. An

outbreak, which may be localized or widespread, similar to an epidemic, may occur when the increase in the incidence of disease is limited in a particular setting. A *cluster* is an aggregate of cases in a given area over a particular period regardless of the number of cases. An epidemic that has spread over several countries or continents and usually affects a large number of people is called a **pandemic**. There are various reasons for initiating and conducting an **outbreak investigation**. One of them is to identify the source so that preventive and control measures that will prevent future episodes of disease are established and instituted. The outbreak investigation also provides the opportunity to learn more about the disease and its severity, the mechanisms for transmission, and the potential for further spread. Although the microbiology laboratory may be first to recognize the event and will likely participate in the outbreak investigation, surveillance data collected by health departments and notifications received by the health department from clinicians who have seen patients with unusual disease presentation may trigger an outbreak investigation.

Local outbreaks

In a given setting, an outbreak may be suspected when an unanticipated increase in infections occurs. Fig. 3.1 shows infection rates that might trigger an outbreak investigation. In this figure, the baseline infection rate is seen for January to August as 3.3 per 1000 catheter days. In addition, the NHSN benchmark of 5.0 per 1000 catheter days is also plotted. In September, the infection rate rose above the baseline and the NHSN benchmark. This might indicate that an outbreak has occurred or that the change in the rate of infections may have other causes. A more detailed investigation would reveal further information.

Case check 3.2

As represented by the Case in Point, an outbreak may also involve the unexpected isolation of a microorganism. During the investigation and introduction of infection control interventions, an epidemiologic curve is created. Fig. 3.2 shows the epidemiologic curve and recovery of the *Acinetobacter* sp. The case first described on the fourth of the month is termed the **index case**, and it was to be determined whether the other infections that followed were related to that case. The investigation used the laboratory results provided by microbiologists.

Widespread outbreaks

Widespread outbreaks occur outside the confines of a given health care setting. These might be found in a statewide or worldwide outbreak (pandemic). An example of a statewide outbreak is the occurrence of diarrheal disease in persons attending a state fair. Fig. 3.3 depicts an **epidemiologic curve** from such an outbreak. This involved reported cases in 565 fair attendees. A total of 47 cultures were obtained, and 26 of those cultures were positive for *Salmonella* spp. Contaminated well water at the fairgrounds was suspected as the source, and increased hand hygiene and use of bottled water were

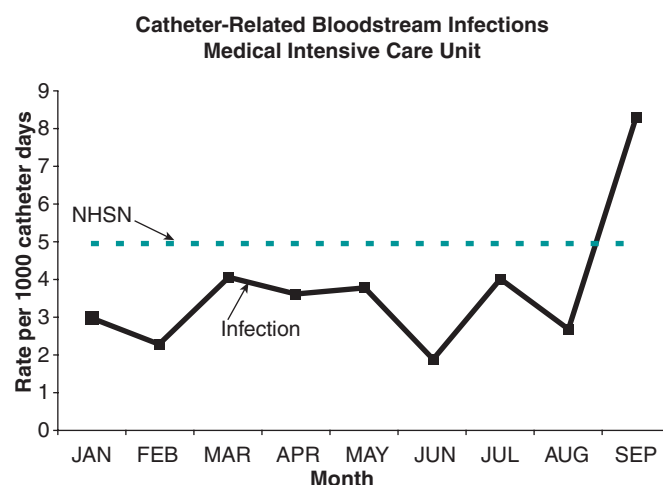


Fig. 3.1 Plot of the rate of catheter-related bloodstream infections in a medical intensive care unit. Shown are the National Healthcare Safety Network (NHSN) benchmark and calculated monthly infection rate. The increase in the rate above the baseline and above the NHSN benchmark in September would trigger an investigation of a possible outbreak.

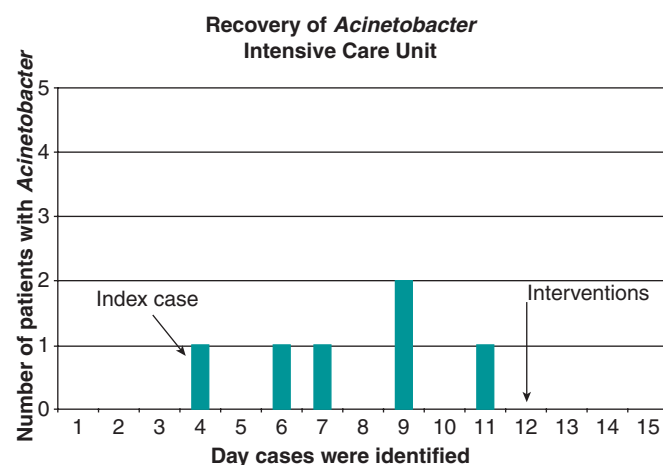


Fig. 3.2 Epidemiologic curve of the occurrence of *Acinetobacter* spp. in respiratory specimens in an intensive care unit. Data were gathered as the result of a suspected outbreak. The graph plots the number of patients with *Acinetobacter* spp. infection by the day on which the cases were identified. Both the index case and the point at which infection control interventions were implemented are indicated on the graph.

instituted 5 days after the first case was reported. After the interventions, the numbers of suspected cases decreased. Cases involved individuals from seven states and Canada. Some were food handlers at the fair in addition to fair attendees. Many suspected cases (>500) were reported but not cultured. The *Salmonella* spp. isolates recovered were all the same serotype. Well water, food, and food preparation sites were cultured.

Another example involves the investigation of a potential measles outbreak involving an international flight from Russia to the United States. The patient suspected of being the index case flew from Russia to London, where she changed planes for a flight to New York. She then changed planes in New York for a flight to Chicago, where she lived. The patient was a college student who had visited her parents in Russia

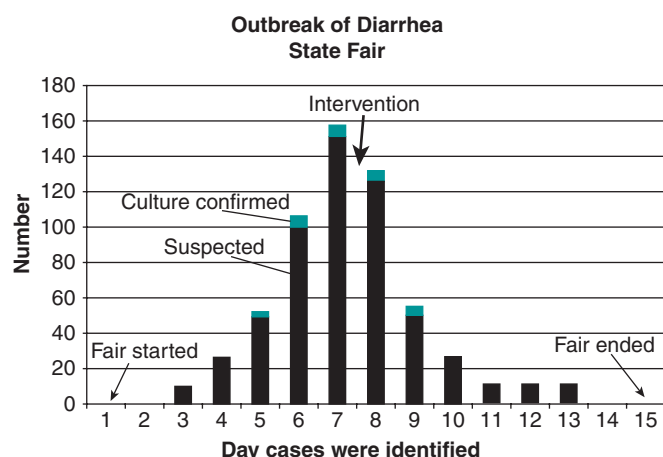


Fig. 3.3 The epidemiologic curve of an outbreak investigation of diarrhea over a 15-day period at a state fair. The number of cases includes suspected cases (black bars) and culture-proven cases (colored bars). Infection control interventions were implemented on day 8 of the outbreak, and the number of cases decreased.

for the summer. While in Russia, she had contact with several young children who had measles. On her return flight to the United States, she complained of a fever but did not develop a rash until she had been home in Chicago for a day.

Imagine the complexity of an investigation of this case. This scenario was recognized as a potential outbreak and not an actual outbreak when the investigation was undertaken. The recognition of measles by the microbiology laboratory set the investigation into motion, not the occurrence of any additional cases. Several issues must be examined.

- Did the student have measles?
- Were passengers on the plane immune?
- Where did the passengers go after their flight landed?
- How does the health department follow up with them?

Because of routine use of a vaccine most, but not all, people in the United States are immune to measles. However, vaccination may not have been successful or given to some individuals. The incubation period for measles is approximately 10 days (8 to 13 days for the fever, 14 days for the rash). People are contagious before the development of the fever and rash. Those who are not immune are susceptible.

All three of these examples (*Acinetobacter* spp., *Salmonella* spp., measles) represent scenarios of potential infectious disease outbreaks. The investigation of these outbreaks depends heavily on the support of the microbiology laboratory to assist in ruling in or ruling out the infection and in identifying cases and sources. Laboratory support is described in detail later.

Pandemic

First identified in December 2019 in Wuhan, China, COVID-19 caused by SARS-CoV-2, a new human pathogen, is a respiratory illness that can be spread from person to person. Cases of COVID-19 spread rapidly around the world. On March 11, 2020, The WHO declared the COVID-19 outbreak a pandemic. To date (19 February 2022), over 422 million cases and nearly 6.55 million deaths have been reported worldwide.

Table 3.4 Steps of an outbreak investigation

Steps	Description
1. Verify diagnosis of suspected cases.	Establish a case definition.
2. Confirm that an outbreak exists.	Be certain that all suspected cases meet the definition.
3. Find additional cases.	Investigate to determine whether additional cases exist.
4. Characterize cases.	Collect as much information as possible about the cases, including people, place, and time elements. Develop an epidemiologic curve.
5. Form a hypothesis.	Establish a "best guess" hypothesis to explain the outbreak.
6. Test the hypothesis.	Evaluate the hypothesis epidemiologically with control groups and data collected.
7. Institute control measures.	Implement intervention activities to prevent and control the outbreak.
8. Evaluate the effectiveness of control measures.	Determine whether the implemented activities have an impact on the outbreak. Does the number of cases diminish or disappear?
9. Communicate the findings.	Document the investigation, and communicate with all involved parties.

In the United States, the first case was identified in a resident of Washington state who had traveled to Wuhan. A remarkable number of cases followed in Seattle and occurred primarily among older adults living in congregant settings such as independent or assisted living communities, long-term care, and skilled nursing facilities. Across the United States, the COVID-19 epidemic has caused several outbreaks that overwhelmed the entire health care system, with the problem growing in scope until mitigation strategies were identified. There have been over 78 million cases and over 900,000 deaths reported in the United States as of February 2022.

Steps of an outbreak investigation

When an outbreak is suspected, steps are taken to investigate the event. The laboratory is integral in several of the steps. [Table 3.4](#) lists the steps that are followed in an outbreak investigation. The first step is to establish a **case definition**. This step ensures that the rest of the investigation is based on a single definition. This may involve the microbiology laboratory in the search for a specific pathogen or the recovery of several pathogens.

The second step is to confirm that an outbreak exists. One needs to be certain that all of the suspected cases match the case definition and that there is more than an expected number of cases. At this point, the investigator seeks as much consultative assistance as possible. The laboratory is frequently asked for additional input.

The third step is to find additional cases that might be added to the initial number of cases. Additional suspected cases may be discovered by more detailed investigation or by the new occurrence of cases. The laboratory might be asked

to review microbiology data from a previous period to determine whether unrecognized cases have occurred.

The fourth step is to gather as much information as possible about the cases with respect to person, place, and time. Persons suspected of being part of the outbreak should be interviewed to find what the victims have in common. An epidemiologic curve may be constructed to assist in the visualization of the outbreak numbers over time.

The fifth and sixth steps are to form a hypothesis about the event and then to test that hypothesis. In the fifth step, a tentative hypothesis is established as a best guess about the likely reservoir, source, and means of transmission. In testing that hypothesis, a control group is established; then the incident and control groups in the event are compared. Again, the microbiology laboratory may provide insight into the hypothesis and its relationship to the control group.

The seventh step in the investigation may actually occur at any point along the investigation timeline. The establishment of interventions to stop the outbreak probably occurs from the initial recognition and heightened awareness of a problem. The formal steps of the intervention process might not be developed until after the hypothesis is developed and tested. Undoubtedly, interventions of some type (e.g., increased hand hygiene) are introduced early in the investigation.

The eighth step, which comes after the development of interventional strategies, is to evaluate the effectiveness of the interventions. Did the outbreak cease or at least decrease in intensity? The ninth step, the final step and one sometimes overlooked, is to communicate the findings of the investigation. This must include a written report that is kept on file and provided to all responsible individuals.

It is not unusual for an outbreak to end before all data have been collected and analyzed. This is probably because of an early intervention. However, an early end to the outbreak does not ameliorate the need for communication and a written report. The information collected can be useful in future outbreaks caused by the same agent.

Investigation support from the laboratory

The microbiology laboratory plays a crucial role in providing investigative support in an outbreak investigation and in the creation of routine surveillance information. The availability of culture reviews, which may result in the initiation of an outbreak investigation, was discussed earlier. Other types of laboratory support are often important as well.

Cultures and serology

In an outbreak investigation and collection of surveillance data, the collection, processing, reporting, and reviewing of pertinent cultures becomes critical. In the *Salmonella* spp. outbreak at the state fair discussed earlier, consider the number of fecal specimens that the health department laboratory processed, although patient cultures are only one component of the investigative activity. Cultures from other sites may provide additional significant information. Well water and food were cultured in addition to specimens from the patients with diarrhea and food handlers. In the outbreak of infections caused by *Acinetobacter* spp., the laboratory may have cultured respiratory therapy equipment and water samples.

One of the major difficulties in a large outbreak (e.g., the state fair outbreak) is the reduced ability to collect and transport specimens from persons from out of state. Individuals may have cultures processed in their home state, but information about these results may be difficult to track and retrieve. In the state fair example, only a few of the affected individuals had cultures processed and the results included in the investigation—565 suspected cases and only 47 cultures. Reasons for this low number of cultures include the following: (1) the diarrhea lasts 24 to 48 hours, and people may not seek medical help; (2) people who live outside the immediate area may not know of the potential outbreak and the need to provide culture material; (3) people do not want to bother with the expense and time spent to collect cultures; and (4) the specimens may be collected too late in the infection or not transported properly, so no organisms are recovered.

In addition to the culture results, the laboratory may be asked to determine the serologic relationship of the isolates.

- Are all the *Salmonella* spp. of the same serotype?
- What is the epidemiologic profile of the serotype?

Both the isolate identification and serologic relatedness may be important determinants in an outbreak investigation.

Antibiograms

Antibiograms, patterns of sensitivity and resistance to antimicrobial agents in bacteria, can often be used in the investigation of an outbreak. As discussed in [Chapter 13](#), there are times when antibiograms must be viewed with suspicion. Although these laboratory data are not always as precise as other results, they may provide guidance about the microbial relatedness of the isolates. [Table 3.5](#) demonstrates comparisons of two antibiograms of isolates with the antibiogram of the index case. If the isolates all had identical antibiograms (e.g., isolate 1) or if some of the isolates were different in their susceptibility patterns (isolate 2), the inclusion or exclusion from the case definitions might be affected. For example, relatively susceptible *S. aureus* can be distinguished from MRSA in an outbreak of skin infections in prisoners.

Table 3.5 Comparison of antibiograms from microbial isolates

Antimicrobial agent	Index case	Isolate	
		1	2
Ampicillin-sulbactam	R	R	S
Piperacillin	R	R	S
Cefepime	R	R	S
Imipenem	S	S	S
Ciprofloxacin	R	R	S
Gentamicin	R	R	S
Tobramycin	R	R	R
Amikacin	R	R	I

I, Intermediate; R, resistant; S, sensitive.

Molecular epidemiology

Molecular epidemiology is the analysis of molecules, such as proteins and nucleic acids, for the detection, identification, and characterization of microorganisms to generate isolate-specific markers to assess epidemiologic relatedness. In identifying SARS-CoV-2 infections, the WHO has endorsed the identification of viral RNA in a patient's biological materials through nucleic acid amplification tests (NAATs) such as reverse-transcription polymerase chain reaction (RT-PCR). A positive result of NAATs targeting at least two different genes, one of which is specific for SARS-CoV-2, provides a final diagnosis of COVID-19. The RT-PCR test remains the "gold standard" for identifying acute SARS-CoV-2 infection. The laboratory has been able to provide a significant and critical contribution to diagnosing an acute SARS-CoV-2 infection through molecular testing. In addition, with serologic testing, the laboratory has been able to detect the presence and extent of an immune response against the virus.

Pulsed-field gel electrophoresis (PFGE) is a strain-typing technique that can be an important adjunct to epidemiologic investigations. In this method, enzyme-digested chromosomal fragments of bacteria are separated electrophoretically. The patterns of the fragments are compared among strains of microbes recovered in a possible outbreak. Strains with dissimilar patterns would be determined to be unrelated. However, if the patterns are similar, the strains can be identified as possibly related. This potential relatedness is an additional epidemiologic tool that can be incorporated into the investigation of an outbreak. PFGE has been used since the 1980s, and it has remained the reference method because of its high discriminatory power, reproducibility, and nearly 100% typeability. A number of other techniques involving primarily amplification of genomic sequences by polymerase chain reaction and gene sequencing are also used to determine relatedness of species in an outbreak.

Environmental culturing

Case check 3.3

As part of an effective IPC program, the microbiology laboratory may be called on to perform cultures of various environmental sites. Recommendations for environmental infection control have been extensively discussed in a CDC document, "Guidelines for Environmental Infection Control in Health-Care Facilities." The environment is rarely implicated in disease transmission, except with immunosuppressed patients. Although environmental cultures are generally to be avoided, as in the Case in Point, there are times when they become an important and often required element of an IPC program.

Air

Usually, infections traced to air quality occur during construction activities in a health care setting. Because microbes in the air can be incriminated in HAIs, cultures of the air can be a component of air quality investigations. Initially, an **infection control risk assessment** should be conducted to determine whether the air is the likely source of infectious particles. Such an assessment is necessary in construction activities and must be done before any decision to culture the air can

be made. The CDC makes no recommendation regarding routine microbiological air sampling before, during, or after construction. If a fungal infection such as aspergillosis occurs during or immediately after construction, an outbreak investigation may be initiated and control measures implemented. Such an investigation may involve collecting environmental samples (e.g., searching for sources of airborne fungi). High-volume air samplers are the preferred method for collection, although settle plates may also be used. The results of these microbiological cultures must be reported to the IPC team and evaluated.

Water

Water is incriminated in outbreaks in many of the settings for which microbiology laboratories provide service. Outbreaks can occur in various environmental situations, such as those associated with contaminated drinking water (e.g., hospitals, ECFs, prisons) or recreational water (e.g., swimming pools, whirlpools, lakes, streams). They may take place in homes, aboard a plane or ship, in a city or state, or in a foreign country. In the United States, an average of 46 waterborne outbreaks were reported each year between 1971 and 2013, resulting in 642,782 outbreak-associated illnesses from 50 states and 6 territories. Waterborne pathogens often cause diarrheal illness. Other waterborne diseases include respiratory illnesses via inhalation of aerosols (e.g., legionellosis), hepatitis (hepatitis A or hepatitis E), skin infections (from *Pseudomonas* spp. or mycobacteria), and central nervous system infections via nasal aspiration (*Naegleria* spp.). Because of these infection control implications, the laboratory must be prepared to offer diagnostic services or recommend laboratories that do offer those services for waterborne pathogens.

Table 3.6 lists examples of waterborne pathogens. Some waterborne agents can be recovered by routine microbiology procedures such as cultures, but others may require specialized techniques. When asked to perform **environmental cultures** of water, the microbiology laboratory must determine which specific pathogens are sought if this information is known. If legionellosis is suspected, for example, the laboratory must have standard operating procedures for recovering this microbe. If the outbreak involves diarrheal diseases of an unknown cause or causes, the recovery techniques must be broader and may require the use of a specialized laboratory. The IPs involved in the outbreak investigation must be consulted before routine culturing of environmental water is undertaken.

Table 3.6 Pathogens related to waterborne infections

Viruses	Bacteria	Parasites
Norovirus	<i>Salmonella</i> spp.	<i>Entamoeba histolytica</i>
Rotavirus	<i>Campylobacter</i> spp.	<i>Giardia lamblia</i>
Hepatitis A	<i>Yersinia enterocolitica</i>	<i>Cryptosporidium</i> spp.
Hepatitis E	<i>Escherichia coli</i> (O157:H7)	<i>Naegleria</i> spp.
	<i>Legionella</i> spp.	<i>Acanthamoeba</i> spp.
	<i>Pseudomonas</i> spp.	
	<i>Mycobacterium</i> spp.	
	<i>Aeromonas</i> spp.	

In some settings, routine water cultures must be performed because of specific guidelines. For example, for chronic dialysis centers, the CDC recommendations include performing bacteriologic assays of water and dialysis fluids at least once a month with standard methods. The IP or managers of the specific area of concern must be familiar with regulations and guidelines addressing water cultures. The laboratory must be aware of the standard methods to ensure that proper procedures and proper media are used. The laboratory scientist, IP, and manager should maintain a close working relationship to ensure compliance with culturing requirements.

Surfaces

The culturing of environmental surfaces should be performed only under the combined direction of the laboratory and IP. In an outbreak investigation, surface culturing may be needed; however, such cultures should not be routinely obtained. The laboratory must be consulted before environmental surface cultures are undertaken to ensure that proper procedures and media are used. The laboratory scientist should be instrumental in the interpretation of the results.

Reporting

The role of the microbiology laboratory does not stop with providing culture results. Depending on statutory requirements, the laboratory might also be responsible for the reporting of certain infectious diseases to public health jurisdictions. Other groups may expect reports as well, such as committees, persons managing specific programs, and the news media.

Reporting to public health

There is a requirement to report the identification or suspicion of certain infectious diseases to local, state, or federal public health entities. As shown in Table 3.7, diseases designated as class A1 are considered public health concerns and must be reported as soon as they are suspected or identified. Some of these requirements are federally mandated, whereas others may be designated by the state. It is imperative that the laboratory scientist knows which infectious diseases are reportable to what agency and in what time frame they are to be reported.

Reporting to committees and programs

Depending on the setting served by the microbiology laboratory, there may be expectations of reports of infectious diseases to committees in that setting. For example, in an acute care hospital, IPC committees may expect reports with various types of microbiological information. Annual antibiograms, lists of reportable diseases, pathogens recovered in certain hospital units, isolates recovered from certain sites, and blood culture contamination rates are examples of reports that might be requested.

In other settings, physicians may expect periodic updates, including antibiograms and pathogen prevalence. They may expect these to be delineated by office practice, physician, site, or patient type. Those making these requests may be

Table 3.7 Examples of reportable diseases^a

Class	Description	Examples
A1	Diseases of major public health concern—reported immediately on recognition of a case, suspected case, or positive laboratory results	Anthrax, botulism (foodborne), cholera, diphtheria, Ebola virus, plague, rabies, smallpox, meningococcal disease, measles, SARS-CoV-2, tularemia, yellow fever
A2	Diseases of public health concern needing timely response—reported by the end of the next business day after recognition of a case, suspected case, or positive laboratory results	Encephalitis (viral), food-borne disease outbreaks, hepatitis A, Legionnaires' disease, pertussis, syphilis, tuberculosis, typhoid fever, vancomycin-resistant <i>Staphylococcus aureus</i> , vancomycin-intermediate <i>S. aureus</i> , tetanus
A3	Diseases of significant public health concern—reported by the end of the work week	Brucellosis, giardiasis, hepatitis B, hepatitis C, Lyme disease, Rocky Mountain spotted fever, trichinosis
B	Diseases reported only by the number of cases—reported by the end of the work week	Chickenpox (varicella), influenza
C	Report of outbreak, unusual incidence, or epidemic—reported by the end of next work day	Blastomycosis, influenza in a health care setting, histoplasmosis, scabies, staphylococcal skin infections, toxoplasmosis

^aThese are reporting regulations from the Ohio Administrative Code and may vary from state to state.

in home health care, extended care, or communal living settings.

Schools and businesses may request updated reports, especially those related to outbreaks that affect their operation. These reports must be tempered by public health needs and individual confidentiality restrictions. Insurance companies and community advocacy groups recently have expressed interest in knowing about infection control rates. Some states require periodic reporting of these rates. The microbiology laboratory may be involved in providing data for these reports.

Reporting to the media

Among the activities of a microbiology laboratory, discussing microbiology and infection control activities with the media (e.g., television, radio, newspapers) may become necessary. Media relations for the laboratory should be discussed with the risk management area associated with the laboratory. Media relations represent an educational opportunity that might be investigated before the laboratory scientist speaks to media personnel. One must balance the public's need for knowledge as perceived by the news media with privacy restraints for the patient, laboratory, and setting.

Education

Laboratory scientists and infection prevention and control practitioners

The role of the microbiology laboratory in education is a further extension of its activities related to infection control. Laboratory scientists must not only keep themselves educated in their contribution to the IPC team, but also keep the infection control personnel educated regarding the laboratory's contribution to the team. Seminars, scientific articles and books, computer-based learning, and discussion with infection control personnel are ways in which laboratory scientists can maintain their knowledge of infection control and laboratory techniques that can aid the infection control program. The laboratorian should continuously educate the IP regarding the abilities of the laboratory in contributing meaningful information to the IPC committee. As new techniques become available or old techniques are replaced, the laboratory scientist must relay this information to the IP.

Similar information must be provided for others associated with the health care setting and IPC program. Ancillary personnel, such as housekeeping and maintenance personnel, benefit from knowing the laboratory perspective of an IPC program:

- What cleaning and disinfecting agents work against viruses?
- How long does the agent need to be in contact with the environment to inactivate the virus?
- When do the maintenance personnel need to worry about fungi?
- What does mold growing on wet drywall look like?

These and other questions arise among ancillary health care personnel, and the laboratory scientist must be prepared to respond.

Consultation with the microbiology laboratory often is sought when construction is anticipated. Sometimes this education must be provided, even when it is not sought.

- Which microbes may be harbored in standing water?
- When should high-efficiency particulate air (HEPA) filters be used?
- What infectious agents might spread through a facility if proper barriers are not used to control demolition dust and debris?

These are some questions that might be addressed by the microbiology laboratory scientist while acting as a consultant to the infection control program.

Safety

The infection control program affects the microbiology laboratory by emphasizing the need for laboratory safety. As discussed in [Chapter 4](#), safety in the microbiology laboratory encompasses the IPC program. Hand hygiene is a critical part of laboratory safety. Hand hygiene involves handwashing when hands are soiled or the use of alcohol hand rubs

Case check 3.4

Whereas the investigation could not conclusively determine why the outbreak in the Case in Point occurred, the use of contact precautions and improved hand hygiene stopped new cases. It is likely that the causative agent, *A. baumannii*, was in the hospital environment and was spread among the patients by improper hygiene. The presence of a ventilator in the affected patients bypassed host defenses in the compromised patients.

when hands are not soiled. The practice of **standard precautions** further extends infection control to the microbiology laboratory. Gloves must always be worn when blood or body fluids are handled. Proper disposal of microbiological waste, according to state or local regulations and national guidelines, is another critical component of the infection control program in the microbiology laboratory. Receiving available vaccines and testing for diseases such as hepatitis B, influenza, meningococcus, and tuberculosis are steps to protect laboratory personnel. All of these functions connect the microbiology laboratory safety program and the infection control program.

Response plans

With the potential use of biological agents in terrorism and emerging pathogens associated with pandemics, the development of **emergency response plans** is paramount. The microbiology laboratory is an integral part of this infection control activity. Safety, specimen collection, agent identification, and agent control are components of the response plan to which the laboratory can contribute its input. Whether in everyday activities or activities in response to an outbreak or emerging diseases, the microbiology laboratory and laboratory scientists constitute a critical component of an efficient and successful infection control program.

POINTS TO REMEMBER

- The microbiology laboratory interacts with the infection preventionist and infection prevention and control committee in many different health care settings.
- Surveillance is important to establish baseline data and recognize the need to investigate potential outbreaks.
- The microbiology laboratory supports outbreak investigations by providing consultative services including epidemiologic correlation of isolates.
- Although infrequently performed, environmental cultures may play a role in outbreak investigation.
- Microbiology laboratory scientists must recognize their role in providing reports to health departments, committees, the infection prevention and control program, and on occasion, the public.
- The microbiology laboratory must maintain competencies in new techniques and the identification of reemerging and emerging infectious diseases.

- In anticipation of potential bioterrorism events and pandemics, the microbiology laboratory must be equipped to participate in emergency preparedness programs.

LEARNING ASSESSMENT QUESTIONS

- Surveillance is defined as:
 - The systematic collection and analysis of data
 - The review of healthcare-associated infections in laboratory personnel
 - The recognition of emerging pathogens
 - The development of an infection control risk assessment
- Microbes commonly encountered in healthcare-associated infections in hospitals include:
 - Salmonella* spp., *Shigella* spp., hepatitis C virus, *Neisseria meningitidis*
 - Staphylococcus aureus*, *Pseudomonas aeruginosa*, MRSA, *Escherichia coli*
 - Pseudomonas aeruginosa*, *Salmonella* spp., hepatitis C virus, *Giardia* spp.
 - All of the above
- Pulsed-field gel electrophoresis (PFGE) might be performed to:
 - Identify staphylococcal species
 - Assist in an outbreak investigation
 - Develop a new isolation precaution
 - All of the above
- The occurrence of surgical site infections is generally calculated as:
 - The rate of infections in 1000 device-related events
 - The percentage of infections in 100 device-related events
 - The percentage of infections in surgical sites or procedures
 - The rate of infections per 100 hospital days
- Health departments frequently require the reporting by the laboratory of:
 - Diseases of major health concerns (e.g., smallpox)
 - Diseases needing timely response (e.g., foodborne outbreaks)
 - Outbreaks of public health concern (e.g., scabies)
 - All of the above
- Microbial pathogens of potential bioterrorism activity include:
 - Bacillus anthracis*, *Staphylococcus aureus*, West Nile virus
 - Yersinia pestis*, *Staphylococcus aureus*, hepatitis C virus
 - Bacillus anthracis*, *Yersinia pestis*, *Francisella tularensis*
 - Bacillus anthracis*, *Escherichia coli*, coronaviruses
- Environmental cultures are usually to be avoided, except in:
 - An outbreak investigation
 - The occurrence of infections following construction
 - Compliance with specific regulatory requirements
 - All of the above
- The formal steps in an outbreak investigation include:
 - Establishing a case definition and culturing air and water
 - Establishing a case definition, forming and testing a hypothesis, and communicating findings
 - Forming and testing a hypothesis, performing PFGE, and calculating an infection rate
 - Confirming an outbreak exists, calculating an infection rate, and performing serology and culture tests
- Infection prevention and control programs rely on microbiology laboratory support in:
 - Public health settings
 - Acute care facilities
 - Home care settings
 - All of the above
- The microbiology laboratory interacts with the infection prevention and control program by providing:
 - Culture results
 - Antibiograms and pathogen prevalence reports
 - Environmental cultures when appropriate
 - All of the above

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4

Control of microorganisms: disinfection, sterilization, and microbiology safety

Michelle M. Jackson*

CHAPTER OUTLINE

Disinfection and sterilization, 66

Sterilization versus disinfection, 66

Factors that influence the degree of killing, 67

- Types of organisms, 67
- Number of organisms 67
- Concentration of disinfecting agent, 68
- Presence of organic material, 68
- Nature of the surface to be disinfected, 68
- Contact time, 68
- Temperature, 68
- pH, 68
- Biofilms, 68
- Compatibility of disinfectants, 68

Methods of disinfection and sterilization, 69

- Physical methods, 69
- Chemical methods, 70

Disinfectants versus antiseptics, 71

- Alcohols, 71
- Aldehydes, 72
- Halogens, 72
- Chlorine and chlorine compounds, 72
- Detergents: quaternary ammonium compounds, 73
- Phenolics, 73
- Heavy metals, 74
- Gases, 74

EPA regulations on chemical surface disinfectants, 75

FDA regulations on chemical skin antiseptics, 75

- Hygienic handwashing and waterless handrubs, 76
- Surgical hand scrub and waterless surgical handrubs, 77
- Presurgical skin disinfection, 77

Microbiology laboratory safety, 77

General laboratory safety, 78

- Safety program for the clinical laboratory, 78
- Hazardous waste, 85
- Chemical safety, 85
- Fire safety, 96
- Storage of compressed gases, 97
- Electrical safety, 97
- Miscellaneous safety considerations, 97
- Safety training, 97

Bioterrorism and the clinical microbiology laboratory, 98

- Laboratory response network, 98
- Safety during a possible bioterrorism event, 98
- Packaging and shipping of infectious substances, 98

Bibliography, 100

OBJECTIVES

After reading and studying this chapter, you should be able to:

1. Define the following terms: *sterilization*, *disinfection*, and *antiseptic*.
2. Differentiate the functions and purposes of a disinfectant and an antiseptic.
3. Describe the general modes of antimicrobial action.
4. Describe the way each physical agent controls the growth of microorganisms.
5. Give the mechanism of action for each type of chemical agent commonly used in antiseptics and disinfectants.
6. Describe the different heat methods and their respective applications.
7. Describe Environmental Protection Agency regulations on chemical surface disinfectants and Food and Drug Administration regulations on chemical skin antiseptics.
8. Discuss the appropriate use of the following skin antiseptics in health care settings by health care personnel: handwash or handrub, surgical hand scrub or surgical handrub, and patient preoperative skin preparation.
9. Describe the hazards that can be encountered in a microbiology laboratory.
10. List the elements included in an exposure control plan.

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11. Discuss the practice of standard precautions.
12. Differentiate standard precautions and transmission-based precautions.
13. Define and give examples of engineering controls, work practice controls, and personal protective equipment.
14. Discuss the World Health Organization classification of infectious microorganisms by risk group.
15. Compare the three types of biological safety cabinets.
16. Differentiate the four categories of biosafety levels.
17. Given an infectious agent and test procedure, justify the appropriate safety precautions to use to ensure that an exposure event does not occur.
18. Explain the information that must be included in safety data sheets.
19. Describe the components of basic fire safety and electrical safety within the microbiology laboratory.
20. Discuss the special safety considerations that must be addressed in the clinical microbiology laboratory during a possible bioterrorism event.

KEY TERMS

Antisepsis	Laboratory response network
Antiseptic	Microbial load
Biofilms	Moist heat
Biological safety cabinet (BSC)	National fire protection association (NFPA)
Biosafety level (BSL)	New drug application (NDA)
Bloodborne pathogens	Over-the-counter (OTC)
Disinfectants	Pasteurization
Disinfection	Patient preoperative skin preparation
Employee right-to-know	Personnel protective equipment (PPE)
Engineering controls	Persistent
Environmental Protection Agency (EPA)	Prions
Exposure control plan	Resident biota (flora)
Fast-acting antiseptic	Risk groups
Filtration	Safety data sheets (SDSs)
Food and Drug Administration (FDA)	Sentinel laboratories
Generally recognized as safe and effective (GRASE)	Sporicidal
Germ theory of disease	Standard precautions
Hazard-rating diamond	Sterilization
Health care antiseptic drug products	Surgical hand scrub
Health care personnel handwash	Transmission-based precautions
Laboratory-acquired infection (LAI)	Transient biota
	Work practice controls

Case in point

Dr. Adams is a geriatrician at a busy metropolitan medical clinic. When she arrives at the clinic, the patients waiting for her in the waiting room are practicing social distancing and wearing masks because of the COVID-19 pandemic. She washes her hands immediately and then uses an alcohol-based hand sanitizer. As she opens the examining room door to see her first patient, Mrs. Jones, the patient notices that Dr. Adams opened the door with her bare hands and is about to examine her. Mrs. Jones tells the doctor to wash her hands first or use the

hand sanitizer before she proceeds with examining her. How should Dr. Adams respond?

Issues to consider**After reading the patient's case history, consider:**

- Potential risks that the physician is taking in spreading infectious agents to her patients
- Importance of handwashing and use of an appropriate antiseptic
- Quality control plan to minimize the risks to patients

Disinfection and sterilization

Safety in the laboratory cannot be overemphasized. Quantification of the risk of working with an infectious agent is difficult. Risk to an individual increases with the frequency and type of organism and level of contact with the agent, as demonstrated by the Case in Point. Each laboratory must develop and institute a plan that effectively minimizes exposure to infectious agents. This chapter provides information on standard disinfection and sterilization techniques for the clinical laboratory. It presents an overview of the following topics:

- Sterilization and disinfection
- Chemical and physical methods of disinfection and sterilization
- Principles and application of each method
- Common disinfectants and antiseptics used in health care settings
- Principles and applications of disinfectants and antiseptics
- Regulatory process of disinfectants and antiseptics

This chapter also covers laboratory safety guidelines for the clinical laboratory practitioner to ensure proper protection and reduce risks of exposure to potentially hazardous biological agents.

Sterilization versus disinfection

The scientific use of **disinfection** and **sterilization** methods originated more than 100 years ago when Joseph Lister introduced the concept of aseptic surgery using carbolic acid, now called *phenol*. Since then, the implementation of effective sterilization and disinfection methods has remained crucial in the control of infections in the laboratory and health care facilities (healthcare-associated infections).

To understand fully the principles of disinfection and sterilization, we must have accurate definitions of certain terms. *Sterilization* refers to the destruction of all forms of life, including bacterial spores. By definition, there are no degrees of sterilization—it is an all-or-nothing process. Chemical or physical methods may be used to accomplish sterilization. The term *sterile* is relevant to the method used. For example, a solution that has been filtered through a certain pore-size filter (<0.22 μm) is often referred to as *sterile*. Even though the filtered solution may be free from large microorganisms

such as bacteria and fungi and their spores, in actuality, any infectious agents (e.g., viruses) that are smaller than the pore size of the filter were not removed; therefore the filtered solution can still contain infectious particles. *Disinfection* refers to a process that eliminates a defined scope of microorganisms, including some spores. Physical or chemical methods may be used, but most **disinfectants** are chemical agents applied to inanimate objects. A substance applied to living tissue (e.g., skin) for the purpose of eliminating or reducing the number of bacteria present is referred to as an **antiseptic**. Antiseptics do not kill spores.

Factors that influence the degree of killing

Before discussing methods used to kill microorganisms, a review of the factors that influence the degree of killing of organisms is important. The following factors play a significant role in the selection and implementation of the appropriate method of disinfection:

- Types of organisms
- Number of organisms
- Concentration of disinfecting agent
- Presence of organic material (e.g., serum, blood)
- Nature of surface to be disinfected
- Contact time
- Temperature
- pH
- Biofilms
- Compatibility of disinfectants and sterilants

Types of organisms

Organisms differ greatly in their ability to withstand chemical and physical treatment (Fig. 4.1). This variability is caused by the biochemical composition of microorganisms and various mechanisms that they can use to protect themselves. For example, bacterial endospores have coats rich in proteins, lipids, and carbohydrates as well

as cores rich in dipicolinic acid and calcium, all of which protect the spores. Cell walls of mycobacteria are rich in lipids, which may account for their resistance to chemical and environmental stresses, particularly desiccation. In contrast, viruses containing lipid-rich envelopes are more susceptible to the effects of detergents and wetting agents. Microorganisms living together in communities, referred to as **biofilms** (see Chapter 31), also provide protection to the microorganisms against chemical and physical means of destruction.

The organisms known today to be the most resistant to the actions of heat, chemicals, and radiation are **prions**. Prions are small, naked infectious proteins. Prions are thought to be the cause of a number of degenerative diseases of the nervous system, e.g., transmissible spongiform encephalopathies ("mad cow disease," Creutzfeldt-Jakob disease). These agents are transmitted to humans through contaminated medicinal products, therapeutic devices, body fluids, and food products. These infectious agents are extremely resistant to chemical and physical methods of destruction. Prions can withstand temperatures exceeding 121°C for several hours while immersed in acid or basic solutions. It is best to handle all body secretions as potentially being contaminated with this agent. When an object or material is thought to be contaminated with a prion, special methods must be taken to destroy the agent. Simple disinfection or sterilization may not be sufficient.

Number of organisms

Another factor to consider is the total number of organisms present, referred to as the **microbial load** (bioburden). If the number of organisms is plotted against the time they are exposed to the killing agent (exposure time) logarithmically, the result is a straight line (Fig. 4.2). The death curve is logarithmic. Because the microbial load is most likely composed of organisms with differing degrees of susceptibility to killing agents, not all of the organisms die at the same time. The microbial load determines the exposure time that is necessary for 99.9% elimination of the microorganisms. In general, higher numbers of organisms require longer exposure times.

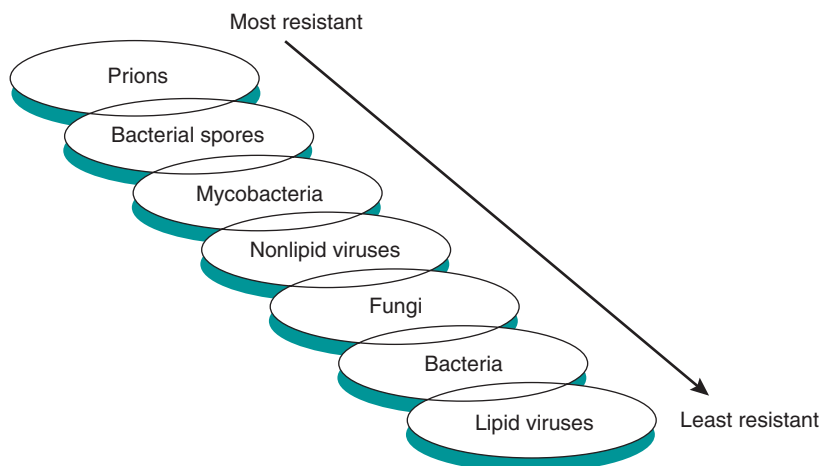


Fig. 4.1 Different types of organisms and their resistance to killing agents.

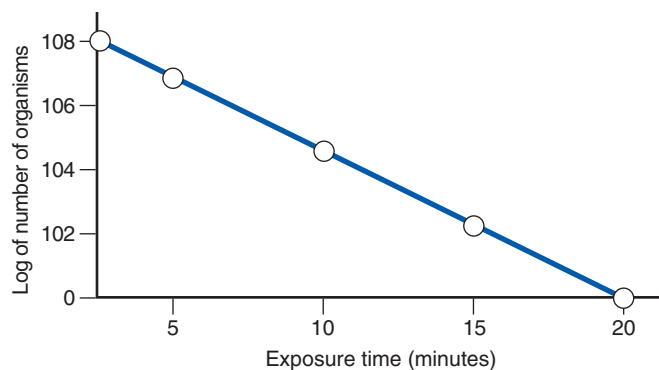


Fig. 4.2 Effect of exposure time versus number of organisms.

Concentration of disinfecting agent

The concentration of a disinfecting agent is also important. The amount of disinfectant needed to destroy microorganisms varies with the different agents. Manufacturers' instructions on preparation, dilution, and use must be followed very carefully. Concentrated disinfectants, such as povidone-iodine, may actually allow microorganisms to survive because there is not enough free iodine to kill microorganisms. Proper concentrations of disinfecting agents ensure the inactivation of target organisms and promote safe and cost-effective practices.

Presence of organic material

Organic material, such as blood, mucus, and pus, affects killing activity by inactivating the disinfecting agent. In addition, by coating the surface to be treated, organic material prevents full contact between the object and agent (see the "Glutaraldehyde" section later in this chapter). Bleach (sodium hypochlorite) is easily inactivated by organic material. For optimal killing activity, instruments and surfaces should be cleaned of excess organic material before disinfection.

Nature of the surface to be disinfected

Certain medical instruments are manufactured of biomaterials that exclude the use of certain disinfection or sterilization methods because of possible damage to the instruments. For example, endoscopic instruments are readily damaged by the heat generated in an autoclave. Alternative methods must be used for this class of instruments.

Contact time

The amount of time a disinfectant or sterilant is in contact with the object is critical. Too little contact time does not allow the agent to work properly. The contact time is a function not only of the agent itself but also of the bioburden on the object, the type of microorganism that is to be killed, and the presence of organic material and the temperature at which the agent is being used. When disinfecting or sterilizing a contaminated object, it is critical to know what organisms may be present and the contact time to use, which is based on the microorganism that is most resistant. The amount of time that an

agent is in contact with an object can also determine whether it is disinfecting or sterilizing the object. For example, glutaraldehyde can be used as a disinfectant or a sterilant, with the difference being the amount of time glutaraldehyde is in contact with the contaminated object. When glutaraldehyde is used as a sterilant, the contact time is much longer than when it is used as a disinfectant. Alcohol and iodine preparations (e.g., Betadine) must be in contact with an object for at least 1 to 2 minutes for them to kill microorganisms. The spores of both bacteria and fungi must be in the presence of disinfectants or sterilants for a much longer time than their vegetative counterpart before they are killed.

Temperature

Disinfectants are generally used at room temperature (20° to 22°C). Their activity is generally increased to some degree by an increase in temperature and decreased by a decrease in temperature. A disinfectant that is used on the workbench generally works faster than if the disinfectant is used on a cold surface such as the walls of a refrigerator. Disinfection of blood spills in a refrigerator can take longer than disinfection of blood spills on a room temperature countertop. Disinfectants and sterilants can be rendered inactive by too high or too low a temperature.

pH

The pH of the material to be disinfected or sterilized can affect the activity of the disinfecting or sterilizing agent. It is critical to make sure at what pH the agent is active and what the pH of the material to be exposed to the agent is at the time the process will be done.

Biofilms

Biofilms are a community of bacteria or other microorganisms. These communities are generally layers of microorganisms that often have a protective material over them that shields them from outside environmental factors. These microbial communities can be on the surface of either inanimate or animate objects. A critical place where biofilms are seen in the hospital is on catheters. Other places may be inside pipes that carry water and on deionizing columns used to make processed water. When dealing with the disinfection of objects that may have a biofilm, it is critical to realize that the presence of the biofilm makes disinfection more difficult. Microorganisms in a biofilm can be characteristically different than when they are planktonic, or singular and free floating. This means disinfectants that the microbes are susceptible to singularly can be resistant within a biofilm (sessile). To disinfect materials that may have a biofilm present, the concentration of the disinfectant may need to be increased, the contact time may need to be increased, or both.

Compatibility of disinfectants

A common mistake is to believe that two disinfectants are better than one. This is not necessarily incorrect, but when more than one disinfectant is used, the compatibility of the disinfectants must be taken into consideration. Some disinfectants may inactivate other disinfectants. For example, the use of

bleach and a quaternary ammonium compound together may negate the activity of both disinfectants.

Whether a chemical agent or physical method is to be used on living tissue or an inanimate object affects the method choice. Clearly, strong, harsh treatments are more effective at inactivating viruses and killing bacteria. However, these treatments would be inappropriate for use as an antiseptic. Bleach is used commonly as a disinfectant to cleanse a laboratory bench, but it is too toxic to be used on the skin as an antiseptic before a venipuncture.

Methods of disinfection and sterilization

Having discussed the way factors that affect the survival of microorganisms influence disinfection and sterilization, we now look at the ways in which methods are selected. E. H. Spaulding categorized medical materials into three device classifications:

1. Critical materials
2. Semicritical materials
3. Noncritical materials

Critical materials are those that penetrate sterile tissues or enter the vascular system. These materials are most likely to produce infection if contaminated, and they require sterilization. Semicritical materials contact mucous membranes, which often contain a normal microbiota. Before these materials are used, they require high-level disinfection agents.

Noncritical materials require intermediate-level to low-level disinfection before contact with intact skin. High-level disinfectants have activity against bacterial endospores, whereas intermediate-level disinfectants have tuberculocidal activity but not **sporicidal** activity. Low-level disinfectants have a wide range of activity against microorganisms but do not demonstrate sporicidal or tuberculocidal activity. [Table 4.1](#) presents a summary of these principles.

Physical methods

As mentioned earlier, sterilization and disinfection can be performed by both physical and chemical methods. Although several physical methods are available, this discussion is restricted to the methods most commonly used in a laboratory or hospital setting.

Heat

Because of its reliable effects, ease of use, and economy, heat is the most common method used for the elimination of microorganisms. Heat can be used in several ways. **Moist heat**, or *heat under steam pressure*, is the principle of autoclaves. Steam under 1 atm of pressure, or 15 psi, achieves a temperature of 121°C. At this temperature, all microorganisms (except for prions) and their endospores are destroyed within approximately 15 minutes of exposure. The time varies according to the density of the material; important factors are that the moisturized heat comes in contact with the material and the contact time is sufficient. An added advantage of moist heat is the shorter time required for sterilization than for dry heat sterilization. Heat in water is transferred more readily

Table 4.1 Device classification and methods of effective disinfection

Device classification	Disinfection method	Killing action against				
		Spores	Mycobacteria	Nonlipid viruses	Fungi	Bacteria
Critical	Sterilization					
	Steam	+	+	+	+	+
	Dry heat	+	+	+	+	+
	Gas	+	+	+	+	+
	Chemical	+	+	+	+	+
	Ionizing radiation	+	+	+	+	+
Semicritical	High-level disinfection					
	2% glutaraldehyde	±	+	+	+	+
	Chlorine dioxide	±	+	+	+	+
	Pasteurization	–	+	+	+	+
	Low-level disinfection					
	Sodium hypochlorite	–	+	±	+	+
	Quaternary ammonium compounds	–	–	±	+	+
	Ethyl alcohol, isopropyl alcohol (60%–90%)	–	–	+	+	+
	Phenolics	–	±	+	+	+
	Iodophors	–	–	+	+	+

+, Positive kill; –, no kill; ±, variable.

to a cool body than heat in air. Moist heat is the sterilization method of choice for heat-stable objects.

Dry heat may also be used as a sterilizing agent, although it requires much longer exposure times and higher temperatures than moist heat. This method can be used for heat-stable substances that are not penetrated by moist heat, such as oils. Dry heat is commonly used to sterilize glassware. Incineration in a flame (e.g., Bunsen burner) or an electric incinerator rapidly destroys infectious agents. However, its use is limited to flame-resistant objects such as metal microbial inoculating loops and needles.

Boiling and **pasteurization** are methods that achieve disinfection but not sterilization; these methods do not eliminate spores. Boiling (100°C) kills most microorganisms in approximately 10 minutes. Pasteurization, used mostly in the food industry, reduces food borne pathogens and organisms responsible for food spoilage. It is generally performed at 72°C (161°F) for 15 seconds. The main advantage of pasteurization is that treatment at this temperature reduces spoilage of food without affecting its taste. [Table 4.2](#) summarizes the applications of heat.

Filtration

Filtration methods can be used with both liquid and air. **Filtration** of liquids is accomplished through the use of thin membrane filters composed of plastic polymers or cellulose esters containing pores of a certain size. The liquid is pulled (vacuum) or pushed (pressure) through the filter matrix. Organisms larger than the size of the pores are retained. Filters with various pore sizes are available. Most bacteria, yeasts, and molds are retained by pore sizes of 0.45

and 0.80 µm; however, this pore size may allow passage of *Pseudomonas*-like organisms and mycoplasma; therefore a 0.22-µm size is available for critical sterilizing (e.g., parenteral solutions). Membranes with pore sizes of 0.01 µm are capable of retaining some viruses. The most common application of filtration is in the sterilization of heat-sensitive solutions, such as parenteral solutions, vaccines, and antimicrobial solutions. Filtration of air is accomplished with the use of high-efficiency particulate air (HEPA) filters. HEPA filters are able to remove microorganisms larger than 0.3 µm and are used in laboratory hoods and in rooms of immunocompromised patients.

Radiation

Radiation may be used in two forms: ionizing and non-ionizing. Ionizing radiation, in the form of gamma rays or electron beams, is of short wavelength and high energy. Ionizing radiation breaks chemical bonds and forms ions damaging and inactivating vital molecules. This method of sterilization is used by the medical field for the sterilization of heat-sensitive disposable supplies such as syringes, catheters, and gloves. Nonionizing radiation in the form of ultraviolet rays is of long wavelength and low energy. It damages deoxyribonucleic acid (DNA) by forming thymine and cytosine dimers. Because of its poor penetrability, usefulness is limited. It can be used to disinfect surfaces, although the parameters (distance to surface, potential microorganisms to be destroyed) under which it is to be used must be determined.

Chemical methods

Just as physical methods are used mainly to achieve sterilization, chemical agents are used mainly as disinfectants. However, some chemical agents may be used for sterilization. These are known as *chemosterilizers*. All disinfectants are regulated by the **Environmental Protection Agency** (EPA). Agents that are classified as *sterilants* are regulated by the **Food and Drug Administration** (FDA) when they are to be used to sterilize devices that will come in contact with patients. Chemical agents exert their killing effect by the following mechanisms:

- Reaction with components of the cytoplasmic membrane
- Denaturation of cellular proteins
- Reaction with the thiol (–SH) groups of enzymes
- Damage of RNA and DNA

It is important to remember that agents can exert one or a combination of actions on microorganisms. Damage to the integrity of the cytoplasmic membrane causes the cytoplasm and its contents to leak out, resulting in cell death. Denaturation of proteins effectively disrupts the metabolism of the cells. Some agents specifically react with the thiol (–SH) groups of enzymes, inactivating them. Thiol groups occur usually in the amino acid cysteine. Finally, damage to RNA and DNA inhibits the replication of the organism or gene expression. For ease of discussion, the chemical agents are grouped on the basis of chemical composition. [Table 4.3](#) summarizes the applications of chemicals commonly used as disinfectants and antiseptics.

Table 4.2 Control of microorganisms using heat methods

Method	Temperature (°C)	Time required	Applications
Boiling water (steam)	100	15 minutes	Kills microbial vegetative forms; endospores survive
Autoclave (steam under pressure)	121.6	15 minutes at 15 psi	Sterilizes and kills endospores
Pasteurization			
Batch method	63	30 minutes	Disinfects and kills milk borne pathogens and vegetative forms; endospores survive
Flash method	72	15 seconds	Same, but shorter time at higher temperature
Oven (dry heat)	160–180	1.5–3 hours	Sterilizes; keeps materials dry

Psi, Pounds per square inch.

Modified from VanDemark, P. J., & Batzing B. L. (1987). *The microbes: an introduction to their nature and importance*. Redwood City, CA: Benjamin-Cummings.

Table 4.3 Chemical agents commonly used as disinfectants and antiseptics

Type	Agent(s)	Action(s)	Applications and precautions
Alcohols (60%–90%)	Ethanol, isopropanol, benzyl alcohol	Denature proteins; make lipids soluble	Skin antiseptics
Aldehydes (in solution)	Formaldehyde (8%), glutaraldehyde (2%)	React with NH_2 , $-\text{SH}$, and $-\text{COOH}$ groups	Disinfectants; kill endospores; toxic to humans
Halogens	Tincture of iodine (2% in 70% alcohol)	Inactivates proteins	Skin antiseptics
	Chlorine and chlorine compounds	React with water to form hypochlorous acid (HClO); oxidizing agents	Used to disinfect drinking water and swimming pools; surface disinfectants
Detergents	Quaternary ammonium compounds	Disrupt cell membranes	Skin antiseptics; disinfectants
Phenolics	Phenol, carbolic acid, Lysol, hexachlorophene	Denature proteins; disrupt cell membranes	Disinfectants at high concentrations; used in soaps at low concentrations
Gases	Ethylene oxide	Alkylating agent	Sterilization of heat-sensitive objects

Modified from VanDemark, P. J., & Batzing B. L. (1987). *The microbes: an introduction to their nature and importance*. Redwood City, CA: Benjamin-Cummings.

Disinfectants versus antiseptics

The **germ theory of disease** was one of the most important contributions by microbiologists to the general welfare of the worldwide population. The medical community gradually grew aware of the problem of nosocomial (hospital-acquired) infections and the need to practice asepsis to prevent the contamination of wounds, dressings, and surgical instruments. The germ theory of disease also contributed to the development of antimicrobial chemotherapeutics.

Ignaz Semmelweis (1816–1865) and Joseph Lister (1827–1912) are considered to be important pioneers for the promotion of asepsis. More than 100 years ago, Semmelweis demonstrated that routine handwashing can prevent the spread of disease. Semmelweis worked in a hospital in Vienna where maternity patients were dying at an alarming rate. Most of the maternity patients who died had been treated by medical students who worked on cadavers during an anatomy class before beginning their rounds in the maternity ward. Because the students did not wash their hands between touching the dead and the living (handwashing was an unrecognized hygienic practice at the time), pathogenic bacteria from the cadavers were transmitted by the hands of the students to the mothers. The result was a death rate five times higher for mothers who delivered in the hospital in contrast with the mothers who delivered at home. Of women who delivered their babies in hospitals, 25% died of childbed fever (puerperal sepsis), later found to be caused by infection with *Streptococcus pyogenes*. In an experiment considered quaint at best by his colleagues, Semmelweis insisted that his students wash their hands before treating the mothers; deaths on the maternity ward were reduced fivefold. Despite these remarkable results, Semmelweis's colleagues greeted his findings with hostility, in part because of the results implying that physicians were unclean. Because of this hostility, he eventually resigned his position. Later in another maternity clinic, he had similar dramatic results when handwashing was implemented. Ironically, Semmelweis died in 1865 of infection with *S. pyogenes*, with his views still largely ridiculed.

After the death of Semmelweis, Lister, an academic surgeon, benefited by reading Pasteur's works about bacteria

as causes of infection before he ventured into studies of antiseptics. In 1867, Lister introduced handwashing and the use of phenol as an antimicrobial agent for surgical wound dressings to British surgery. His principles were gradually, although reluctantly, adopted in Britain, and the mortality rate for amputation decreased from 45% to 15%. The Listerian technique was approved in the United States 20 years after Semmelweis's initial publications at the first official meeting of the American Surgical Association in 1883. This was the beginning of infection control. Health care workers' hands are frequently contaminated by direct contact while caring for a patient or by indirect contact while touching a contaminated surface or device.

Alcohols

The two most effective alcohols used in hospitals for disinfection purposes are ethyl alcohol and isopropyl alcohol. Alcohols have excellent in vitro bactericidal activity against most gram-positive and gram-negative bacteria. They also kill *Mycobacterium tuberculosis* and various fungi, and they inactivate certain enveloped viruses. However, they are not sporicidal and have poor activity against nonenveloped viruses. Because alcohols are not sporicidal, alcohol may actually be contaminated with spores. Any solution of an alcohol that is used as an antiseptic or disinfectant should be filtered through a 0.22- μm filter to remove spores that may be present. In addition, because alcohols are not sporicidal, when alcohol is used to saturate cotton balls to be used to prepare the skin for blood collection or inoculation, the cotton balls should be sterile. Today, alcohols used as an antiseptic are sold as sterile, individually wrapped pads.

Alcohols are inactivated by the presence of organic material. Because their action is greatly reduced in concentrations less than 50%, alcohols should be used in concentrations between 60% and 90%. For alcohols to be effective, they must be given time to evaporate from the surface to which they were applied. Alcohols inactivate microorganisms by denaturing proteins, dehydrating cells, and dissolving lipids. Alcohols are used as antiseptics and disinfectants. They are used to disinfect laboratory surfaces and gloved hands, as well as skin surface before venipuncture, injection, or

inserting an intravenous needle. They may be used as a housekeeping disinfectant for damp-dusting furniture and lights or wiping electrical cords without leaving a residue on treated surfaces. They are nonstaining and can disinfect semi-critical instruments. Alcohols are flammable, so they should not be used in the presence of ignition sources such as open flames or incinerators.

The 2017 final rule for **health care antiseptic drug products** defers ethanol (60% to 95%) from inclusion in the final rulemaking to allow more time for completion of studies necessary to fill the data gaps needed for health care personnel handwash, surgical hand scrub, and patient preoperative skin preparation (for preparation of the skin before injection). After reviewing currently available scientific evidence, the FDA tentatively determined that there are important scientific data gaps for health care antiseptic products marketed under the previous 1994 tentative final monograph (TFM) proposed rule to support the products' effectiveness at reducing infections and meet safety requirements. The 2015 proposed rule states that more extensive data than previously proposed in the 1994 TFM are necessary to demonstrate that **over-the-counter (OTC)** monograph topical antiseptics used in the health care setting are **generally recognized as safe and effective** (GRASE). At the present time, the FDA is evaluating additional safety and efficacy of alcohols for use as health care antiseptics.

Aldehydes

Formaldehyde

Formaldehyde is generally used as formalin, a 37% aqueous solution, or formaldehyde gas. Formaldehyde gas is often used to disinfect **biological safety cabinets** (BSCs). This type of procedure should be performed by professionals. Although formalin can be used as a chemosterilizer in high concentrations, its usefulness is limited by its irritability factor and its potential carcinogenicity. Formaldehyde is a carcinogen, and the U.S. Occupational Safety and Health Administration (OSHA) has set worker exposure limits. It is not recommended that formaldehyde in any form be used as a disinfectant or sterilant on a routine basis. *M. tuberculosis* has been known to survive for many years in tissue fixed in formaldehyde.

Glutaraldehyde

Glutaraldehyde, classified as a high-level disinfectant, is a saturated five-carbon dialdehyde that has broad-spectrum activity and rapid killing action and remains active in the presence of organic matter. Glutaraldehyde is extremely susceptible to pH changes and is active only in an alkaline environment. When used as a 2% solution, it is germicidal in approximately 10 minutes and sporicidal in 3 to 10 hours. Its killing activity is caused by inactivation of DNA, RNA, and proteins through alkylation of sulfhydryl and amino groups. Even though glutaraldehyde is not inactivated by organic material, it does not penetrate organic material well. Therefore objects coated with organic material should be cleaned before glutaraldehyde is used. Glutaraldehyde solutions can be reused; however, a decrease in the activity of the glutaraldehyde may be seen as a result of accumulation of organic material, dilution, and change in the pH of the solution. Because it does not corrode

lenses, metal, or rubber, it is the sterilizer of choice for medical equipment that is not heat-stable and cannot be autoclaved and for material that cannot be sterilized with gas. It is safe, for most plastic and rubber items, such as items used for administering anesthetic agents or respiratory therapy.

Glutaraldehyde is bactericidal, pseudomonacidal, fungicidal, and virucidal (against human immunodeficiency virus [HIV] and hepatitis B virus [HBV]) with a minimum of 10 minutes exposure at a temperature between 20° and 30°C. It is also tuberculocidal. Variations in formulations of products available affect the exposure time and temperature of the solution, especially after reuse. A 2% solution at 25° to 30°C may be 100% tuberculocidal. Users should follow the label instructions of the manufacturer. Most of the products labeled as *cold sterilants* are sporicidal in a minimum of 10 hours exposure at room temperature.

Halogens

Iodophors

Iodine can be used in one of two forms: tincture or iodophor. Tinctures are alcohol and iodine solutions, used mainly as antiseptics. An iodophor is a combination of iodine and a neutral polymer carrier that increases the solubility of the agent. This combination allows the slow release of iodine. Iodophors must be diluted properly for them to be effective. Improperly diluted iodophors may not kill microorganisms because of the lack of free iodine in solution. Iodophors have the added advantage of being less irritating, nonstaining, and more stable than iodine in its pure form. Iodophors may be used as antiseptics or disinfectants, depending on the concentration of free iodine. The best known iodophor is povidone-iodine (Betadine), which is mainly used as an antiseptic. Povidone-iodine provides slow and continuous release of free iodine. Free iodine degrades microbial cell walls and cytoplasm, denatures enzymes, and coagulates chromosomal material.

Iodophors are commonly used as skin preparation agents for sites where blood is to be drawn for blood cultures. When iodophors are used for this purpose, it is critical that there is the proper amount of contact time, which is generally more than 30 seconds. All iodine tinctures and iodophors must be completely removed from the skin to avoid irritation. Iodophors are used only to disinfect because they are not sporicidal. Their bactericidal action is caused by the oxidative effects of molecular iodine and hypoiodic acid, both of which are found in solution. The FDA has deferred povidone-iodine 5% to 10% from inclusion in the final rulemaking to allow more time for completion of studies necessary to fill the data gaps for health care personnel handwash, surgical hand scrub, and patient preoperative skin preparation. At the present time, the FDA is evaluating safety and efficacy data of iodophors for use as health care antiseptics.

Chlorine and chlorine compounds

Chlorine and chlorine compounds are some of the oldest and most commonly used disinfectants. They are usually used in the form of hypochlorite, such as the liquid sodium hypochlorite (household bleach) and solid calcium hypochlorite.

Their killing activity is based on the oxidative effects of hypochlorous acid, which is formed when chloride ions are dissolved in water. Hypochlorites are inexpensive and have a broad spectrum of activity; however, they are not used as sterilants because of the long exposure time required for sporicidal action and their inactivation by organic matter. Hypochlorites are corrosive; therefore concentrated bleach solutions should not be used for disinfection. The activity of hypochlorite solutions is greatly influenced by the pH of the surrounding medium.

These solutions are commonly used as surface disinfectants (e.g., for tabletops). A solution containing 0.5% to 1% sodium hypochlorite is generally used for disinfection. Such solutions are generally stable for no longer than 30 days, with 50% of the original concentration of chlorine dissipating by 30 days. However, current practice recommends making a fresh dilution daily. As with the use of any disinfectant, proper contact time is critical for bleach solutions. Solutions should be allowed a contact time of at least 3 minutes, and longer if organic material is present. A 1:10 dilution of a 5.25% concentration of sodium hypochlorite is recommended by the Centers for Disease Control and Prevention (CDC) for cleaning blood spills. The most common use of chlorine is disinfection of water.

Detergents: quaternary ammonium compounds

Quaternary ammonium compounds are derived by substitution of the four-valence ammonium ion with alkyl halides. They are cationic, surface-active agents (surfactants) that work by reducing the surface tension of molecules in a liquid. Their effectiveness is reduced by hard water and soap, and they are inactivated by excess organic matter. Their action is mediated through disruption of the cellular membrane, resulting in leakage of cell contents. Certain bacteria, particularly gram-negative bacteria such as *Pseudomonas aeruginosa*, are intrinsically resistant to quaternary ammonium compounds. *Pseudomonas* spp. growing in ammonium acetate-containing quaternary ammonium compounds and disease outbreaks associated with contaminated quaternary ammonium compounds have been reported. Because they are not sporicidal or tuberculocidal, the use of quaternary ammonium compounds is limited to disinfection of noncritical surfaces such as tabletops and floors.

Phenolics

Phenolics are molecules of phenol (carbolic acid) that have been chemically substituted, typically by halogens or alkyl, phenyl, or benzyl groups. These groups reduce the toxicity of phenol and increase its effectiveness. The most common phenolics are *ortho*-phenylphenol and *ortho*-benzyl-*para*-chlorophenol. Phenolics have a fairly broad spectrum of activity but are not sporicidal. The addition of detergents to the phenol formulation makes products that clean and disinfect in one step. They are stable, biodegradable, and relatively active in the presence of organic material. Their mechanism of inactivation is disruption of cell walls, resulting in precipitation of proteins. At lower concentrations, phenolics are able to disrupt enzyme systems. Their main use is in the disinfection

of hospital, institutional, and household environments. They are also commonly found in germicidal soaps.

Chlorhexidine gluconate

Chlorhexidine gluconate (CHG) has been used for more than 30 years in the hospital setting. In 1976, the FDA granted approval of CHG for use as a topical antiseptic on the basis of its high level of antimicrobial activity, low toxicity, and strong affinity for binding to the skin and mucous membranes. CHG was not an OTC drug monograph active ingredient at that time. CHG disrupts the microbial cell membrane and precipitates the cell contents. CHG is broad spectrum, being active against gram-positive and gram-negative bacteria. It has less activity against fungi and tubercle bacilli. CHG is bacteriostatic (inhibits bacterial growth) at low concentration and bactericidal (kills bacteria) at high concentration. CHG is inactive against bacterial spores except at elevated temperatures. Lipid-enveloped viruses (e.g., herpesvirus, HIV, influenza virus, cytomegalovirus) are rapidly inactivated. Nonenveloped viruses (e.g., rotavirus, adenovirus, enteroviruses) are not inactivated by CHG.

Numerous studies indicate that CHG is safe and nontoxic. It is not absorbed through the skin and has a low skin-irritancy potential. However, severe skin reactions can occur in infants younger than 2 months of age. The potential for allergic contact sensitization and photosensitization is reported to be minimal. However, CHG should not come into contact with the eyes, the middle ear, or meninges. Although CHG is not as rapidly effective as the alcohols, a major attribute of CHG is its persistence in that it binds to the skin and remains active for at least 6 hours. It is not significantly affected by organic matter such as blood and is pH-dependent—hence the formulation significantly affects activity. The optimum pH range of 5.5 to 7.0 corresponds to the pH of body surfaces and tissues.

CHG is used extensively for disinfection of the hands of surgical personnel and provides whole-body disinfection of patients undergoing surgery. Low-concentration (0.5% to 1%) CHG is added to alcohol-based preparations to provide greater residual activity than alcohol alone. The immediate bactericidal action of CHG surpasses that of antiseptic preparations containing povidone-iodine, triclosan, hexachlorophene, or chloroxylenol. Its persistence, which prevents regrowth of microorganisms on the skin, is comparable to that of hexachlorophene or triclosan. CHG has a broader spectrum of activity than the others, especially against gram-negative bacteria.

Hexachlorophene

Hexachlorophene is primarily effective against gram-positive bacteria. It is a chlorinated bisphenol that interrupts bacterial electron transport, inhibits membrane-bound enzymes at low concentrations, and ruptures bacterial membranes at high concentrations. Gram-positive bacteria are killed by 3% hexachlorophene within 15 to 30 seconds, but a longer time is needed for gram-negative bacteria. Hexachlorophene has residual activity for several hours after application and has a cumulative effect after multiple uses. Hexachlorophene has been associated with severe toxic effects, including death. It can be absorbed through damaged skin of adults and the skin of premature infants. The FDA classified 3% hexachlorophene

to be available only by prescription and designated it as unsafe for OTC distribution. Hexachlorophene is indicated to control outbreaks of gram-positive infections when other infection-control procedures have been unsuccessful. Hexachlorophene should be used only as long as necessary for infection control.

Chloroxylenol

Chloroxylenol (parachlorometaxylenol [PCMX]) is a halogen-substituted phenolic compound that has been used in the United States since the 1940s. PCMX at concentrations of 0.5% to 4% acts by microbial cell wall disruption and enzyme inactivation. PCMX has good activity against gram-positive bacteria, but it is less active against gram-negative bacteria, *M. tuberculosis*, fungi, and viruses. The antimicrobial activity of PCMX is unaffected by organic materials such as blood or sputum, but it is neutralized by nonionic surfactants and polyethylene glycol. It is considered intermediate-acting to slow-acting and has minimal **persistent** effect of more than a few hours. PCMX has low antimicrobial efficacy compared with iodines, iodophors, and CHG in reducing skin biota. The FDA has deferred PCMX (0.24% to 3.75%) from inclusion in the final rulemaking to allow more time for completion of studies necessary to fill the data gaps for health care personnel handwash. The FDA is currently evaluating additional safety and efficacy of PCMX for use as a health care antiseptic under the OTC drug review.

Triclosan

Triclosan is a diphenyl ether that disrupts the cell wall. The reaction time is intermediate, and the persistence is excellent. It has good activity against gram-positive bacteria, gram-negative bacteria, and viruses. It has fair activity against *M. tuberculosis* but poor activity against fungi. Triclosan is not significantly affected by organic matter such as blood, but it is affected by pH and the presence of surfactants and emollients, and formulation significantly affects activity. Triclosan can be absorbed through intact skin. Some short-term animal studies have shown that exposure to high doses of triclosan is associated with a decrease in the levels of some thyroid hormones. The FDA currently does not know the significance of these findings to human health. Other studies have raised the possibility that exposure to triclosan contributes to making bacteria resistant to antimicrobial agents. The FDA currently does not have enough information available to assess the level of risk that triclosan poses for the development of antimicrobial resistance. The FDA published a final rule for consumer antiseptic hand and body washes and health care antiseptics containing the majority of the antibacterial active ingredients, including triclosan and triclocarban, that they will no longer be able to be marketed.

Manufacturers have not proven that triclosan is safe for daily use over a long period. Additionally, manufacturers have not shown that this ingredient is any more effective than plain soap and water in preventing illness and the spread of certain infections. Triclosan has been added to many consumer products, including clothing, kitchenware, furniture, toothpaste, and toys to prevent bacterial contamination. The EPA regulates the use of triclosan in these products and is presently reevaluating its assessment of the effects of triclosan when used in these products.

Heavy metals

Disinfectants containing heavy metals are rarely used in clinical applications. They have been replaced by safer and more effective compounds. Heavy metal disinfectants are slowly bactericidal; their action is primarily bacteriostatic. Because of the toxic effects of mercuric chloride and other mercury compounds, their use as disinfectants has declined, and they are used mainly as preservatives for paint. Silver nitrate (1% eye drop solution) had been used as a prophylactic treatment to prevent gonococcal (*Neisseria gonorrhoeae*) conjunctivitis in newborns. It has been largely replaced by erythromycin drops. Copper and copper alloys (e.g., brass, bronze) are the first solid surface materials ever to receive approval by the EPA to be considered antimicrobial for health care use. They are being evaluated for use lining rails and door handles in health care facilities to mitigate the spread of microorganisms.

Gases

Ethylene oxide

Ethylene oxide is the gas most commonly used for sterilization. Because it is explosive in its pure form, it is mixed with nitrogen or carbon dioxide before use. Factors such as temperature, time, and relative humidity are extremely important in determining the effectiveness of gas sterilization. The recommended concentration is 450 to 700 mg of ethylene oxide per liter of chamber space at 55° to 60°C for 2 hours. A relative humidity of 30% is optimal for the destruction of spores. The killing mechanism of ethylene oxide is the alkylation of nucleic acids in the spore and vegetative cell. Gas sterilization is widely used in hospitals for materials that cannot withstand steam sterilization. This method is also used by the manufacturing industry for the sterilization of low-cost thermoplastic products. However, its use has declined in the last few decades because of health concerns and time needed for sterilization.

Hydrogen peroxide

Vaporized hydrogen peroxide (H_2O_2) is primarily used as a sterilant in the pharmaceutical and medical device manufacturing industries. It is active against all vegetative microorganisms, bacterial endospores, and fungal spores. In a 3% to 6% liquid form, it can be used as an antiseptic with an intermediate to high activity level. Hydrogen peroxide can be inactivated by bacterial peroxidases and catalases.

Peracetic acid

Peracetic acid is used as a gaseous sterilant primarily in the pharmaceutical and medical device manufacturing industries. It is active against all vegetative microorganisms and bacterial and fungal spores.

Hydrogen peroxide and peracetic acid

The combination of H_2O_2 and peracetic acid vapors is used in the pharmaceutical and medical device manufacturing industries. Similar to each of its individual components, the combination of H_2O_2 and peracetic acid is active against all

vegetative forms of microorganisms and bacterial and fungal spores. The major advantage to the use of the combination of H_2O_2 and peracetic acid over each individual component is a shorter contact time. The activity against prions of the combination of H_2O_2 and peracetic acid as well as each of the individual components is not fully known.

EPA regulations on chemical surface disinfectants

The Antimicrobial Division of the EPA regulates the registration on the use, sale, and distribution of antimicrobial pesticide products for certain inanimate, hard nonporous surfaces or incorporation of antimicrobial pesticide products into substances under the pesticide law—the Federal Insecticide, Fungicide, and Rodenticide Act. An EPA registration number is granted only when the requirements of laboratory test data, toxicity data, product formula, and label copy are approved. The label of a disinfectant that is effective against a specific major group of microorganisms only (e.g., gram positive or gram negative) must specify the major group against which it is effective. Label claims of effectiveness as a “general disinfectant” or representations that the product is effective against a broad spectrum of microorganisms are acceptable if the product is effective against gram-positive and gram-negative bacteria. Label claims for use of disinfectants in hospital or medical environments are acceptable only for products that are effective as general or broad-spectrum disinfectants as well as disinfectants against the healthcare-associated bacterial pathogen *P. aeruginosa*. The disinfectant label should indicate several highlighted points important in selecting the appropriate agents for the designated use (Box 4.1).

BOX 4.1 Type of information to review on a disinfectant label

Front panel

- Product name, brand, or trademark
- Ingredient statement (concentration or strength)
- “Keep Out of Reach of Children”
- EPA registration number and establishment number

Back panel

- Precautionary statements
- Hazards to humans and domestic animals
- First aid
- Environmental hazard
- Physical or chemical hazard
- Directions for use
- How to use the product
- Application sites and rates
- Worker protection issues
- Aftercare
- Equipment
- Treated surfaces
- Cleaning supplies
- Storage and disposal

EPA, Environmental Protection Agency.

FDA regulations on chemical skin antiseptics

When developing an antiseptic drug product, a manufacturer can pursue two options: the **new drug application (NDA)** process or the OTC drug review known as the *monograph system*. NDAs are defined by law as being recognized as safe and effective. A new chemical entity never before marketed in the United States would be classified as a new drug and, in most cases, initially approved for prescription use only. The approved NDA is manufacturer-specific and allows only that particular sponsor to market the product. Other manufacturers wanting to market a similar product would also need to seek FDA approval through an NDA. The FDA considers a drug safe enough to approve when the benefits outweigh the risks. This risk-to-benefit assessment is critical in the drug-approval process.

OTC drugs are defined as GRASE for their intended use as long as they are neither misbranded nor marketed using false or misleading statements. In March 2020, the Coronavirus Aid, Relief, and Economic Security Act (the CARES Act) ushered in a wave of reforms to modernize the regulation of OTC drugs in the United States. Many of the new provisions had immediate effect. For example, replacement of the notice-and-comment rulemaking process for OTC drug monograph or Tentative Monograph are deemed GRASE under the CARES Act. These existing monographs, with some caveats, are being converted into final administrative orders. Administrative orders allow for faster agency decisions as the process can be run through the FDA instead of being required to go through the rulemaking process with issuance of a final rule (as required by the old OTC drug review process). The new system is meant to help streamline the process and provide a more efficient and effective review.

A manufacturer desiring to market a monographed (therapeutic classes of ingredients that are GRASE) drug does not need to seek clearance from the FDA before marketing the drug. In this case, marketing is not exclusive, and all data and information supporting GRASE status are publicly available. Monographs mainly address active ingredients in the product, and final formulations are not subject to monograph specifications in most cases. Manufacturers are free to include any inactive ingredients that serve a pharmaceutical purpose as long as those ingredients are considered safe and do not interfere with product effectiveness or required final product testing. In some instances, although the product may contain GRASE ingredients, the final formulation may need to meet a monograph testing procedure. An example would be the antiseptic drug products that are for **health care personnel handwash, surgical hand scrub, and patient preoperative skin preparation**. Table 4.4 lists terms and definitions frequently used for topical antiseptics in health care settings. These products are required to meet in vivo and in vitro efficacy testing requirements to ensure that the formulated products are effective as an antiseptic. Inactive ingredients and emollients can inhibit the antiseptic action when included in the products; therefore testing must be performed to show effectiveness.

Table 4.4 Food and Drug Administration product categories of topical antiseptics

Category	Definition
Antiseptic drug	Representative of a drug, in its labeling, as an antiseptic shall be considered to be representation that it is a germicide, except in the case of a drug purporting to be, or represented as, an antiseptic for inhibitory use as a wet dressing, ointment, dusting powder, or such other use as involves prolonged contact with the body
Broad-spectrum activity	Properly formulated drug product, containing an ingredient included in the monograph, that possesses in vitro activity against the microorganisms listed in §333.470(a)(1)(ii), as demonstrated by in vitro minimum inhibitory concentration determinations conducted according to methodology established in §333.470(a)(1)(ii)
Health care antiseptic drug product	Antiseptic-containing drug product applied topically to the skin to help prevent infection or help prevent cross contamination
Antiseptic handwash or health care personnel handwash drug product	Antiseptic-containing preparation designed for frequent use; it reduces the number of transient microorganisms on intact skin to an initial baseline level after adequate washing, rinsing, and drying; it is broad-spectrum, fast-acting, and, if possible, persistent
Surgical hand scrub drug product	Antiseptic-containing preparation that significantly reduces the number of microorganisms on intact skin; it is broad-spectrum, fast-acting, and persistent
Patient preoperative skin-preparation drug product	Fast-acting, broad-spectrum, and persistent antiseptic-containing preparation that significantly reduces the number of microorganisms on intact skin
From Federal Register on the tentative final monograph for health-care topical antiseptic drug products (June 17, 1994, 59 FR 31402).	

Hygienic handwashing and waterless handrubs

The main goal of handwashing is to eliminate **transient biota**. Transient biota is contracted from the environment or from other people. In most cases, these organisms are not part of the established normal biota.

Case check 4.1

The Case in Point exemplifies the significance of handwashing in the prevention of disease and pathogen transmission. Routine handwashing in health care settings is performed in the following situations:

- Removing physical dirt (including blood, excretions, secretions, or discharge from lesions)
- Before and after routine patient contact
- After contact with infected or colonized patients or their immediate surroundings
- In high-risk units such as intensive care and burn units
- On entering protective isolation units and leaving source isolation units
- Before antiseptic procedures (e.g., dressing techniques, minor invasive procedures)

For example, the health care worker in the Case in Point may acquire infectious agents, including methicillin-resistant *Staphylococcus aureus* (MRSA) and coronavirus-2 (CoV-2), during direct contact with patients or contaminated surfaces (e.g., doorknobs). Hands should always be washed immediately after touching an object. Although the health care worker's hands appeared to be clean, her hands harbored many bacteria and infectious microorganisms after having contact with the contaminated doorknob. She should have either washed her hands or used hand sanitizer as soon as she entered the room.

According to the CDC, washing hands with plain soap and water is the best way to avoid being infected and to prevent

the spread of infections. If soap and water are not available, the CDC recommends using an alcohol-based hand sanitizer that contains at least 60% alcohol to reduce the number of organisms on hands. However, hand sanitizers do not eliminate all types of bacteria, are not as effective when hands are visibly dirty or greasy, and may not remove harmful chemicals. Hand sanitizers are an easy, quick alternative when handwashing with plain soap and water is not convenient or possible. Hand hygiene should be a topic of conversation between the health care provider and the patient. Health care providers should not hesitate in discussing how and why they cleanse their hands before, after, and sometimes during patient care, and they should inform the patient that it is okay to ask about hand hygiene. Although transient organisms are easily removed from the upper layer of the skin along with dirt particles and oil, they may become part of the **resident biota** of individuals. Interventions against the bacterial load of the hands should balance two goals: (1) protecting the skin with its resident biota and (2) killing the transient biota. Intact skin on health care workers' hands helps protect both patients and health care workers from getting or transmitting healthcare-associated infections.

The FDA requires that all antiseptic handwashing and handrubbing products used in the hospital setting reduce the number of sampled test bacteria by a superiority margin (compared with a negative control) of $1.2 \log_{10}$ for handwash and $1.5 \log_{10}$ for handrub on each hand within 5 minutes after the first wash/rub application. The technique involves treating the hands with the antiseptic product according to the manufacturer's instructions for the specified time. The lower third of the forearm is also washed. After completion of the wash, hands and forearms are rinsed under tap water ($40^\circ\text{C} \pm 2^\circ\text{C}$) and dried thoroughly with disposable or sterilized towels.

Waterless handrubs (e.g., alcohol handrubs)—either liquid or gel—are used for hygienic hand antiseptics. They also can be used as an alternative to routine handwashing when there is no visible soiling and for patient contact. They are often more convenient than handwashing and can be particularly

useful if sinks are not readily available. Dispensers for alcohol handrubs can be placed next to sinks and beside each bed or carried by health care providers. The technique involves rubbing small portions (3 to 5 mL) of a **fast-acting antiseptic**, usually an alcoholic preparation, into the hands and rubbing until dry or for a preset duration recommended by the manufacturer. All areas of the hands must be covered completely with the antiseptic, including the subungual spaces of the fingers.

Surgical hand scrub and waterless surgical handrubs

The objective of the surgical hand scrub and waterless surgical handrubs is to eliminate transient biota and most of the resident biota. Resident biota can be persistently isolated from the hands of most people. These organisms include coagulase-negative staphylococci, *Corynebacterium* spp. (diphtheroids or coryneforms), *Propionibacterium* spp., *Cutibacterium*, and *Acinetobacter* spp.

The rationale is to limit bacterial exposure of the surgeon's hands in case the surgical glove is punctured or torn. Tiny holes are observed in 30% or more of surgeons' gloves after an operation, even when high-quality gloves are used. A surgical hand scrub drug product is defined as an antiseptic containing a preparation that significantly reduces the number of microorganisms on intact skin; it is broad-spectrum, fast-acting, and persistent. The FDA requires that the product reduce on the first wash/rub the number of bacteria by a superiority margin of $0.5 \log_{10}$ for hand scrub and $1.5 \log_{10}$ for handrub on each hand within 1 minute after application and that the count on each hand does not subsequently exceed the baseline within 6 hours after product use. Surgical hand scrub procedures are performed according to the manufacturer's instructions, but for no longer than 5 minutes. They offer the advantage of cleansing and disinfecting the hands at the same time.

Surgical handrubs, alcohol solutions (60% to 70% ethyl alcohol or propyl alcohol) with an emollient and with or without an added antiseptic as recommended for hygienic waterless handrubs, are used, but larger volumes and longer exposure times are needed than for hygienic waterless handrubs. Surgical handrub application technique is accomplished by pouring a specified amount of antiseptic into the cupped dry hands and rubbing vigorously all over the hands and forearms, which must be kept wet with the handrub solution for the period of 3 to 5 minutes by adding additional portions as necessary and continuing to rub. Before the application of an alcohol, the hands must be dry, and the alcohol must have completely evaporated before donning gloves.

Presurgical skin disinfection

To be effective, preoperative skin preparation formulations must degerm an intended surgical site rapidly as well as provide a high level of bacterial inactivation and persistent antimicrobial activity, up to 6 hours after preparing the skin. A patient preoperative skin preparation drug product is defined as a fast-acting, broad-spectrum, and persistent antiseptic-containing preparation that significantly reduces

the number of microorganisms on intact skin. Similar to the surgeon's hands, the patient's operation site requires surgical disinfection, directed against resident as well as transient biota, and it often requires maximum disinfection in a single treatment, without the benefit from progressive effects of repeated application.

The FDA requires that the product reduce the number of bacteria by a superiority margin of $1.2 \log_{10}$ per square centimeter on an abdomen and groin test site within 30 seconds or 10 minutes after product use and that the bacterial count for each test site does not exceed the baseline within 6 hours after product use. For preinjection sites, the product must reduce the number of bacteria by a superiority margin (compared with a negative control) of $1.2 \log_{10}$ per square centimeter on a dry skin test site within 30 seconds after product use. It has long been debated whether or not a preoperative skin preparation is adequate in degerming the skin before making a surgical incision. Many surgeons recognize this problem, and to promote greater degerming of the surgical site area, patients are requested to wash the area daily, or more often, before surgery. The intent is to reduce the microbial population at the surgical site area so the region is prepared before surgery. The organisms, then being few in number, would be virtually totally removed from the skin.

Microbiology laboratory safety

Case in point

A 35-year-old infectious disease specialist complains of experiencing fever, chills, myalgia, and severe headache for the past several days. The patient has been otherwise healthy until 2 weeks ago, when he began to experience fatigue and slightly swollen cervical lymph nodes. The patient recalls that he demonstrated to his medical students and residents a culture of *Brucella melitensis* isolated from a patient's blood sample. While handling the Petri dish without gloves, he picked up the telephone to answer a page. He then touched his mustache while talking on the telephone, as he habitually does when he speaks.

Issues to consider

After reading the patient's case history, consider:

- Potential risks to which laboratory scientists are exposed while working in the microbiology laboratory
- Exposure control plan to minimize the risks
- Laboratory safety guidelines to protect laboratory personnel

While laboratory safety procedures have improved greatly over the last few decades, safety in the clinical laboratory is a major concern, and work practices must be continually reevaluated. Studies have shown that laboratorians are at increased risk of infections compared with the general population. Laboratory exposures do occur, and often they cannot be traced back to a specific event. In 2012, the CDC published "Guidelines for Safe Work Practices in Human and Animal Medical Diagnostic Laboratories." This report was developed to improve safety in the laboratory by increasing safe practices, to encourage laboratorians to consider safety issues, and to foster a culture of safety. Safety in the clinical laboratory

encompasses biological, chemical, electrical, radioactive, and fire hazard protection.

General laboratory safety

Safety in the clinical laboratory is the responsibility of the institution, laboratory directors, laboratory managers, and laboratory employees. Laboratory employees must be provided with a safe work environment and necessary **personal protective equipment (PPE)**. OSHA defines PPE as specialized clothing or equipment that is worn by an employee for protection. Laboratory directors, managers, and employees must know the current safety regulations; safety procedure manuals must be provided; and training in safe laboratory practices must occur on an annual basis through in-service education and should be the duty of an assigned safety officer. Although the provision of a safe work environment is ultimately the employer's responsibility, it cannot be achieved without the commitment of all persons in that environment to practice safe techniques for their own and their coworkers' protection.

Case check 4.2

The Case in Point at the beginning of this section illustrates the importance of a safety program for the clinical laboratory. Safety in the laboratory is the responsibility of all laboratory personnel. Everyone who enters the laboratory must also observe the safety guidelines to ensure proper protection and reduce risk of exposure to potentially hazardous biological agents.

Safety program for the clinical laboratory

The comprehensive safety program for the clinical laboratory must fulfill the following:

- Address biological hazards by performing biological risk assessments and developing safety procedures for working with these hazards.
- Describe the safe handling, storage, and disposal of chemicals and radioactive substances.
- Clearly outline the laboratory or hospital policies for correct procedures in the event of fire, natural disasters, and bomb threats.
- Perform initial safety training for all employees in all aspects of laboratory safety and update training annually.
- Teach correct techniques for lifting and moving heavy objects.

The safety program must be ongoing and consistent with current federal and state regulations. Most important, it must be presented in a way that encourages employees to incorporate the safety practices into their daily routines and take responsibility for keeping the work environment safe.

Occupational Safety and Health Administration

Laboratorians must always remember that they work in a hazardous environment. Hazards can be classified as biological, chemical, radiologic, or physical. Training programs are instituted in all of these areas for employees who encounter any of these hazards. It is imperative for

individuals to follow the rules that are set forth in the safety procedure manuals.

The mission of the OSHA is to protect workers within the United States. The clinical laboratory falls under these regulations. In 1991, the OSHA created and released the Bloodborne Pathogens Final Standard to protect health care workers. This standard was revised in 2001 in conformance with Public Law 106-430, the Needlestick Safety and Prevention Act.

Exposure control plan

The OSHA Bloodborne Pathogens Final Standard clearly states the safety requirements that the employer must have in place to protect employees from **bloodborne pathogens**. Employers are required to have an **exposure control plan**, which must be reviewed and updated annually. This plan must be available to all employees and should include the following:

- A determination of tasks and procedures that may result in an occupational hazard
- A plan to investigate all exposure incidents and a plan to prevent these from recurring
- Methods of compliance for standard precautions
- Engineering and work practice controls
- PPE required
- Guidelines for ensuring that the work site is maintained in a clean and sanitary manner
- Guidelines for the handling and disposal of regulated waste
- A training program for all employees

Standard precautions

In 1985, the CDC instituted safety guidelines for the handling of blood and body fluids. These guidelines, called *universal precautions*, were intended to protect hospital personnel from bloodborne infections. In 1996, these guidelines were updated and renamed. These new guidelines, **standard precautions**, are still in effect. These guidelines require that blood and body fluids from *all* patients be considered infectious and capable of transmitting disease. Blood and all body fluids, including secretions and excretions except sweat, regardless of whether visible blood is present, are considered infectious. Standard precautions also include nonintact skin and mucous membranes.

To ensure that the guidelines required in standard precautions are followed within the laboratory, engineering controls and work practice controls are instituted, and employers must provide PPE. Standard precautions address the following:

- *Handwashing* must be done after touching blood, body fluids, secretions, excretions, and any items considered contaminated. Hands must be washed after removal of gloves and between patients.
- *Gloves* should be worn when handling blood, body fluids, secretions, excretions, and any items considered contaminated. Clean gloves must be put on before touching mucous membranes and nonintact skin. Hands must be washed after removal of gloves.
- *Mask, eye protection, or face shield* must be worn anytime there is a potential for splashes or sprays of blood, body fluids, secretions, or excretions.

- *Laboratory coats* must be worn to protect skin and clothing when contact with blood, body fluids, secretions, or excretions could occur.
- *Appropriate sharps disposal* must be implemented with care to prevent injuries with sharps, needles, and scalpels. These devices must be placed in appropriate puncture-resistant containers after use.
- *Environmental control* must include procedures for routine care, cleaning, and disinfection of environmental surfaces.

Transmission-based precautions

The second set of precautions for the health care setting are called **transmission-based precautions**. Standard precautions are still followed, and transmission-based precautions are added precautions that are used when the patient is known or suspected to be infected or colonized with an infectious agent that requires extra measures to prevent spread or transmission of the agent. The categories of these precautions are contact precautions, droplet precautions, and airborne precautions. Contact precautions are used to stop the spread of infectious agents that can be transmitted through direct or indirect contact with the patient or with the patient's environment. Examples of these types of infectious agents are multidrug-resistant organisms such as vancomycin-resistant enterococci, MRSA, and *Clostridioides difficile*.

Droplet precautions are used to stop the spread of infectious agents that can be transmitted by close respiratory contact or by exposure of mucous membranes to respiratory secretions. Examples of infectious agents that can be transmitted by this route include *Neisseria meningitidis*, *Bordetella pertussis*, influenza virus, and CoV-2. The final category is airborne precautions. These precautions are used for infectious agents, such as *M. tuberculosis*, varicella-zoster virus, and rubeola virus, that can remain airborne and infectious over long distances. For further information on what safety procedures should be used for each category of precautions, refer to the 2007 (Updated 2019) *Guideline for Isolation Precautions: Preventing Transmission of Infectious Agents in Health care Settings* published by the CDC.

Engineering controls

Engineering controls are defined by the OSHA as controls that isolate or remove the hazard from the workplace. Examples of engineering controls include the use of closed tube sampling by laboratory equipment and the use of safety needles and single-use holders, eyewash stations, emergency showers, and plastic shield barriers. Ideally, laboratories should have negative air pressure, access to the laboratory should be limited, and there should be a plan to prevent insect infestation.

Work practice controls

Altering the manner in which a task is performed to reduce the likelihood of exposure to infectious agents is defined by the OSHA as **work practice controls**. Examples of work practice controls include the following:

- No mouth pipetting
- No eating, drinking, smoking, or applying cosmetics in the laboratory
- Disinfection of workstations at the end of each shift and after any spill of infectious material
- No recapping or breaking of contaminated needles
- Disposal of needles in an appropriate puncture-resistant container
- Procedures performed in a manner that minimizes splashing and the generation of air droplets
- Specimens transported by way of well-constructed containers with secure lids to prevent leakage of infectious materials
- Frequent handwashing

Personal protective equipment

PPE must be provided and maintained by the employer; examples include gloves, laboratory coats, masks, respirators, face shields, and safety glasses. For PPE to be protective and considered appropriate, blood and body fluids must not be able to penetrate the PPE material. The equipment must be accessible to the employee and must be worn whenever there is the potential for exposure to infectious material. It must be removed before leaving the work area and must be placed in an area designated for PPE. Gloves should be removed whenever they become contaminated, and disposable gloves should *never* be washed and reused. Hands must be washed after the removal of gloves.

PPE must fit properly to be the most effective. Respirators that are used for protection against airborne transmission of infectious agents must be fit-tested to ensure the protection of the worker. Fig. 4.3 illustrates goggles, masks, and laboratory garments appropriate for use in laboratory workstations.

Biological risk assessment

For an infection to occur, including an LAI, there must be a susceptible host, the infectious agent must have a route of transmission to the susceptible host, and the concentration of the agent must be high enough to cause disease. The biological hazards in the microbiology laboratory come from two major sources: (1) processing of the patient specimens and (2) handling of the actively growing cultures of microorganisms. Either activity puts the employee at risk of potential contact with infectious agents. The major routes of LAIs in the clinical laboratory are parenteral inoculations (e.g., needlesticks or contaminated sharps), spills and splashes onto skin or mucous membranes, ingestions (e.g., putting pens or fingers into the mouth), and inhalation of infectious aerosols. Families of microbiology personnel and persons who work in adjacent laboratories may also be at risk.

Many infectious agents pose a high risk to laboratory employees. *M. tuberculosis* has long been known to cause tuberculosis in laboratory workers exposed to aerosols created in processing sputum samples. A laboratory spill of active *M. tuberculosis*, which could easily aerosolize through the ventilation system, is of great concern. *Brucella* spp. and *Francisella tularensis* are other infectious agents that can be transmitted through inhalation of an aerosol created while processing or handling specimens (e.g., blood that may harbor these organisms) or cultures of the organism. *Coccidioides immitis*, an infectious fungus, can infect several people in a room if culture plates on which the organism is growing are opened in the absence of a BSC. In the 1970s, *Bacillus anthracis*



Fig. 4.3 **A**, Eye protection used when chemical splashing could occur. **B**, Integrated eye protection and mask. **C**, Laboratory coats. The material of the white laboratory coat slows the penetration of liquids that splash or soak it. The coat on the *right* is disposable.

caused the deaths of a laboratory worker's wife, who handled his contaminated laboratory coat, and their infant. Emerging pathogens, such as CoV-2, pose a major risk because there is often a lack of knowledge and experience among those working with these emerging infectious agents.

Bloodborne pathogens also pose a high risk. HBV can be transmitted to laboratory workers through needlestick injuries or cuts from other sharp instruments that have been contaminated with an infected patient's blood or body fluids or through contact with mucous membranes. Historically, health care workers have had a higher seropositivity rate for HBV than the general population. The CDC stated that before the institution of the hepatitis B vaccine in 1982, more than 10,000 health care workers were accidentally infected with this bloodborne pathogen annually. By 2001, the number had decreased to less than 400. HIV and hepatitis C virus are other bloodborne pathogens that may

be transmitted to laboratory personnel from contaminated specimens through a needlestick injury or another percutaneous route. All of these infectious agents must be handled with extreme care.

Because microbiology laboratory personnel frequently deal with various infectious agents—viral, fungal, bacterial, parasitic, and mycobacterial—LAIs are an obvious hazard and should be a concern for all laboratorians. Laboratorians must understand that they are at risk of an LAI because of the environment in which they work, and it should be the goal of laboratory management and laboratory workers to minimize this risk and follow all of the safety procedures employed by the laboratory for their protection. An example of the risk to laboratorians and their family members of LAIs was an outbreak of *Salmonella* Typhimurium infections. Between August 2010 and June 2011, 109 individuals were infected with this organism in over 38 states. An epidemiologic study identified

one possible link—exposure to microbiology laboratories, including teaching laboratories and clinical laboratories. The CDC, along with other organizations, identified areas where biosafety and laboratory safety improvements should be made. Some of the recommendations for students, laboratorians, laboratory directors and managers, and faculty included the following:

1. Laboratorians and students should know that bacteria handled in the laboratory can make people sick. These organisms can also make family members sick. Individuals must not take items into the laboratories that will be taken home, such as laboratory coats, pens, books, laboratory report forms, cell phones, and keys.
2. Students should have dedicated writing utensils and supplies at their workstations, and these should not leave the laboratory.
3. Laboratorians and students must be aware of the organisms with which they are working and what the signs and symptoms are if they become infected with one of these organisms.
4. Laboratorians and students must be educated and proficient in biosafety practices and techniques.
5. Laboratory coats should always be worn over personal clothing (appropriate PPE should be worn). Individuals should not leave the laboratory with PPE on; PPE always should be disposed of properly.
6. Handwashing sinks and supplies must be provided. Laboratorians and students should always wash their hands before leaving the laboratory.

Biological risk assessment is an important part of every microbiology laboratory safety program. Biological risk assessment is a process used to recognize the hazardous characteristics of infectious agents that may be encountered in the clinical microbiology laboratory. Also included in the risk assessment process are the laboratory practices that could result in an infectious exposure, the likelihood that an LAI will occur, and the consequences of that infection. Through this process, appropriate safety practices can be identified to protect laboratorians.

The hazardous risk characteristics of an agent are determined by the agent's ability to infect and cause disease in humans or animals; its virulence; the availability of treatment for the disease; and whether there are any preventive measures, such as a vaccine, that can be used to prevent disease by the agent. The World Health Organization (WHO) defined four **risk groups** for infectious agents based on the hazardous characteristics listed previously. The four risk groups, defined in the WHO classification of infectious microorganisms, correlate with the *biosafety levels* (BSLs) discussed later in this chapter, but are not always equal (Box 4.2). Other factors that must be considered when one is determining the correct BSL needed are the mode of transmission, the microbiological procedures that will be performed, and the experience of the staff members. A biological risk assessment is a comprehensive attempt to determine what controls should be used to protect the worker and environment from exposure. It is a subjective process, and different strategies can be used to perform the assessment. Five steps have been outlined in the CDC's "Guidelines for Safe

BOX 4.2 Classification of infective microorganisms by risk group

Risk group 1 (no or low individual and community risk)

A microorganism that is unlikely to cause human or animal disease.

Risk group 2 (moderate individual risk, low community risk)

A pathogen that can cause human or animal disease but is unlikely to be a serious hazard to laboratory workers, the community, livestock, or the environment. Laboratory exposures may cause serious infection, but effective treatment and preventive measures are available, and the risk of spread of infection is limited.

Risk group 3 (high individual risk, low community risk)

A pathogen that usually causes serious human or animal disease but does not ordinarily spread from one infected individual to another. Effective treatment and preventive measures are available.

Risk group 4 (high individual and community risk)

A pathogen that usually causes serious human or animal disease and that can be readily transmitted from one individual to another, directly or indirectly. Effective treatment and preventive measures are not usually available.

From World Health Organization. (2020). *Laboratory biosafety manual* (4th ed.). Geneva, Switzerland: World Health Organization.

Work Practices in Human and Animal Medical Diagnostic Laboratories" for performing a risk assessment. These are as follows:

1. Identify the hazards associated with an infectious agent or material.
2. Identify the activities that might cause exposures to the agent or material.
3. Consider the competencies and experience of laboratory personnel.
4. Evaluate and prioritize risks (evaluate the likelihood that an exposure would cause an LAI and the severity of consequences if such an infection occurs).
5. Develop, implement, and evaluate controls to minimize the risk for exposure.*

Processing of patient specimens

Labeling only specimens from patients with known hepatitis or acquired immunodeficiency syndrome (AIDS) and requiring "extra precautions" for dealing with these specimens does not provide adequate protection for laboratory workers. Many patients come to the emergency department or are admitted to the hospital with no diagnosis. These patients may be in the early stages of either disease or may be asymptomatic but still contagious. Because the incidence of hepatitis and AIDS is relatively high, the likelihood of exposure to one or both of these bloodborne pathogens is elevated. For

*Centers for Disease Control and Prevention. (2012). Guidelines for safe work practices in human and animal medical diagnostic laboratories *MMWR Suppl* 61(01), 1. Available at: https://www.cdc.gov/mmwr/preview/mmwrhtml/su6101a1.htm?s_cid=su6101a1_w.

this reason, standard precautions should be used when handling all patient samples.

When samples are received in the laboratory, often not enough information is available to assess the risk involved with processing the sample. Laboratory scientists do not know what infectious agents are present in the sample and will not know until the agent or agents have been identified. For this reason, it must be assumed that the specimen contains agents that correlate with at least a BSL-2, unless there is additional information suggesting that there may be an agent present from a higher-risk group. A common practice is to perform all specimen processing in a BSC because of the uncertainty regarding the infectious agents that might be present in the sample.

Case in point

A medical laboratory scientist is reading cultures at the bench. During her work, she isolates and identifies *Shigella sonnei*. She starts having watery diarrhea 48 hours later, which progresses to abdominal cramps and bloody stools. Because of her signs and symptoms, her primary care practitioner orders a stool culture.

Issues to consider

After reading the patient's case history, consider:

- The identity of the organism causing the infection
- The potential for an LAI
- The importance of laboratory safety procedures

Working with actively growing cultures

Many of the guidelines in place for the protection of microbiology personnel against exposure to bloodborne pathogens also apply to working with cultures of microorganisms at the bench. Some of the safety precautions that should be used in the microbiology laboratory are the following:

1. Hands must be **washed** frequently and kept away from the nose, mouth, and eyes. Hands should be washed after removal of gloves.
2. Appropriate PPE should be worn.
3. Adhesive bandages or small finger cots should be worn directly over cuts or hangnails.
4. Plates should not be waved in front of the face to determine the odor of the organism.
5. Splash guards are recommended for any procedure that may produce splashes, such as work at the blood culture bench. Gram-negative coccobacilli from a blood culture should be handled in a BSC.
6. Do not use open flames in the laboratory.
7. Any plates growing a fungus should immediately be sealed, and the culture should be worked on in a BSC.
8. Any cultures suspected of growing other potentially aerosolized infectious agents, such as *M. tuberculosis* or *Brucella* spp., should be worked on only in a BSC.

In an effort to educate individuals at risk of LAI, the CDC publishes a manual entitled *Biosafety in Microbiological and Biomedical Laboratories* every few years, which contains important information on the degree of risk of biological agents for laboratory workers and the safety procedures that should be used when working with infectious agents.

Case check 4.3

S. sonnei was isolated from the stool culture of the laboratory scientist from the Case in Point. The laboratory supervisor investigated the incident. It was discovered that the laboratory scientist had her cell phone with her in the laboratory and used the phone while working at the laboratory bench. She stated that at the end of her shift she wiped the phone with alcohol. It appeared that the LAI occurred because the laboratory scientist did not follow the safety protocols in the microbiology laboratory.

Biological safety cabinets

BSCs are a form of engineering control that is used throughout the microbiology laboratory. These hoods are a type of containment barrier that protects the worker from the aerosolized transmission of infectious agents. Any procedure that has the ability to create aerosols should be performed in a BSC. Microbiology clinical samples should also be handled within a BSC. There are three types of BSC: class I, class II, and class III (Fig. 4.4).

A BSC must be maintained, inspected, and used properly, and it is imperative that the laboratory worker understand the functions and limitations of the unit. The most common type of BSC used in a microbiology laboratory is the class II. Box 4.3 provides a few guidelines to follow to ensure the proper use of a BSC.

Biosafety levels

LAI may occur through error, accident, or carelessness. However, the mode of transmission is unknown in many LAIs. For this reason, strict guidelines have been instituted to protect the worker during all laboratory tasks. Earlier in this chapter, the classification of infectious microorganisms into risk groups was discussed. When considering which BSL to use, the laboratorian should know the risk group for the agents being handled, the mode of transmission for the agents, and the procedures that will be performed (e.g., whether aerosols will be created). The *Biosafety in Microbiological and Biomedical Laboratories* manual from the CDC recommends that the BSLs should be used when working with particular agents and performing certain procedures. Four BSLs with guidelines were established for each level for protection.

Biosafety level 1

Infectious agents that would be classified as requiring BSL-1 containment are agents that are well classified and are not known to consistently cause disease in healthy adults. These agents pose a minimal threat to laboratory personnel and

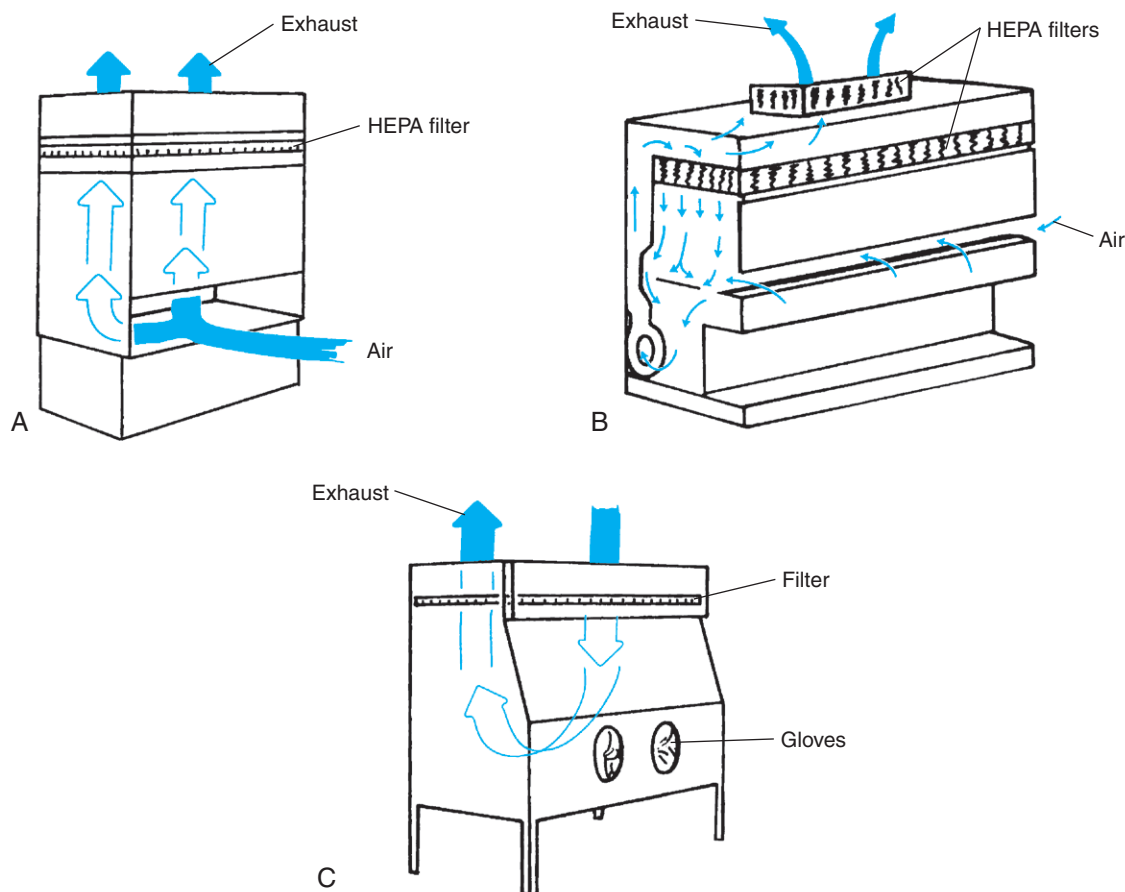


Fig. 4.4 **A**, The class I biological safety cabinet (hood) uses an exhaust fan to move air inward through the open front. The air is circulated within the safety hood, passing through a high-efficiency particulate air (HEPA) filter before reaching the environment outside the hood. **B**, The class II biological safety cabinet is the most common in microbiology laboratories. Air is pulled inward and downward by a blower and passed up through the air flow plenum, where it passes through a HEPA filter before reaching the work surface. A percentage of the remaining air is HEPA filtered before reaching the environment. **C**, The class III biological safety cabinet is a self-contained ventilated system for highly infectious microorganisms or materials and provides the highest level of personal protection. The closed front contains attached gloves for manipulation on the work surface. *Note:* Chemical fume hoods cannot be used as biological safety cabinets, and biological safety cabinets cannot be used as chemical fume hoods.

BOX 4.3 Proper use of a biological safety cabinet (BSC)

- Be aware of the functioning and limitations of the unit. You and your specimen are being protected solely by an air curtain barrier, and anything that disrupts this curtain threatens your safety. Ensure that the cabinet has been inspected within the past year and that the air pressure readings across the HEPA filter are within specifications.
- Plan your work, anticipating the order of events.
- Turn on the incandescent lights and the blower fan. Be sure the UV light is off. Wait 15 to 30 minutes to ensure satisfactory establishment of the air curtain.
- Wash your hands, and then gown and glove. Wear a mask or other personal protective equipment as appropriate.
- Decontaminate the interior work surfaces by cleaning them thoroughly with 70% ethanol.
- Organize all necessary work materials, and place them in the cabinet. Do not place any extra items in the cabinet. Ensure that both the front intake grill and the rear-wall or floor exhaust grills are unobstructed.
- Segregate clean and contaminated items, and place them to minimize subsequent movement with the BSC. The discard bucket/pan should be near the rear of the cabinet but not obstructing the exhaust grill.
- Do not use open flames in the unit. Instead, use disposable supplies or an incinerator.
- All arm movements into and within the cabinet should be slow and deliberate so as to minimize disruption of the air curtain. Allow adequate time at the conclusion of movement for the air curtain to reestablish itself.
- When work is completed, remove all nonpermanent items from the BSC, and allow the cabinet fans to continue running for at least 30 minutes to ensure thorough filtering of the inside air (assuming the fans are not left on permanently). Turn on the UV lights to disinfect the interior of the cabinet.

BSC, Biological safety cabinet; HEPA, high-efficiency particulate air; UV, ultraviolet.

Data from Kruse, R. H., Puckett, W. H., & Richardson J. H. (1991). Biological safety cabinetry. *Clinical Microbiology Reviews*, 4(2), 207–241.

the environment. Safety guidelines for handling these agents include the following:

1. Access to the laboratory is limited or restricted, and there must be a biohazard sign posted at the entrance of the laboratory.
2. Employees must wash their hands after they have removed their gloves, after they have handled live organisms, and before they leave the laboratory.
3. Employees must follow basic work practice controls.
4. Employees must follow OSHA guidelines for handling needles and sharps.
5. Work surfaces must be decontaminated after completion of work and any time there has been a spill of potentially infectious material.
6. Laboratory coats and gloves should be worn and other PPE, such as face shields and eye protection, may be needed when there is a potential for splashes or sprays of infectious or other hazardous materials.
7. Cultures and stock material must be decontaminated before disposal.
8. An insect and rodent control plan must be in effect.
9. The laboratory facility should be designed so it can be easily cleaned. Carpets and rugs should not be used. The laboratory must be equipped with handwashing sinks, an eyewash station, and adequate illumination.

Laboratory work can be conducted on open bench tops in a BSL-1 laboratory. Employees should be educated in laboratory procedures and supervised by a scientist with training in microbiology or a related science. Standard microbiology practices should be followed at all times. Examples of BSL-1 organisms include *Bacillus subtilis* and *Klebsiella aerogenes*.

Biosafety level 2

Infectious agents that require BSL-2 containment and practices are agents that pose a moderate potential hazard for the employees and the environment. The guidelines are the same as for BSL-1 with added precautions for BSL-2 agents as follows:

1. Employees in the laboratory must have specific education in the handling of pathogenic agents and should receive annual updates or additional training as needed.
2. Access to the laboratory is limited when work is being conducted. The laboratory director is ultimately responsible for determining who may enter or work in the laboratory.
3. Laboratorians should receive immunizations or tests for agents handled or for agents that could potentially be in the laboratory environment (e.g., hepatitis B vaccination and the tuberculin skin test).
4. A biosafety manual must be developed and updated.
5. A BSC must be used whenever there is a potential for creating infectious aerosols or splashes or when high concentrations of infectious agents are used. The recommended BSC is class II.
6. All PPE must be worn. Gloves must be worn to protect hands from exposure to hazardous materials.
7. Extreme precautions are taken with contaminated sharp items. The use of needles and syringes is restricted within the laboratory to only times when there is no alternative equipment that can be used.

Examples of BSL-2 organisms include HBV, HIV, *Salmonella* spp., and *Toxoplasma gondii*.

Biosafety level 3

BSL-3 containment and practices are required for infectious agents that are either indigenous or exotic. These agents have the potential for aerosol transmission, and diseases with these agents can have serious lethal consequences. The guidelines for BSL-2 laboratories must be followed along with the more stringent guidelines needed to handle BSL-3 agents safely. Laboratory personnel must have specific training in handling of these pathogenic and potentially lethal organisms. A few of the special BSL-3 guidelines are as follows:

1. The handling of infectious materials, samples, and cultures must occur within a BSC or other physical containment device.
2. Personnel must wear appropriate PPE. Gloves must be worn to protect hands from exposure to hazardous materials.
3. The BSL-3 laboratory should be separated from the other parts of the building and should be accessed through two self-closing doors. An anteroom may be used for access.
4. The BSL-3 laboratory requires a ducted air ventilation system that must provide sustained directional air flow. This directional air flow pulls air from “clean” areas toward “potentially contaminated” areas (negative air pressure).
5. The ceilings and floors must be solid, and any seams must be sealed.
6. All parts of the laboratory must be constructed for easy cleaning and decontamination.

Examples of BSL-3 agents include *M. tuberculosis*, St. Louis encephalitis virus, *Coxiella burnetii*, and CoV-2 (the cause of COVID-19).

Biosafety level 4

BSL-4 containment and practices are required when working with agents that are dangerous and exotic. These agents have a high risk of causing life-threatening infections, can be transmitted by aerosols, or have an unknown risk of transmission. Vaccines are typically not available. As in all microbiology practices, specific training is required. Laboratory personnel must receive thorough training in the handling of these dangerous agents, and they must be trained in how to use the containment barriers that are in place for protection. The BSL-4 facility either is located in a separate building or is in an isolated zone within a building. This facility is isolated from all other areas, and access is strictly controlled. The guidelines listed for BSL-3 must be followed along with additional guidelines for handling BSL-4 agents. There are two types of BSL-4 laboratories: cabinet and suit. In the cabinet laboratory, all work is performed within a class III BSC. In a suit laboratory, personnel wear a positive pressure protective suit to perform all work. Some of the additional guidelines are as follows:

1. Access is strictly controlled, and the supervisor has the ultimate responsibility for who has access. A logbook is maintained to document all personnel who enter and exit the laboratory.

2. As required by all levels, a biohazard sign must be posted outside the door with the potential hazards; the laboratory director's name; and any special requirements, such as immunizations, for entering the area.
3. All personnel must demonstrate high proficiency in standard and special microbiology practices.
4. Policies and procedures are established on the collection and storage of serum samples from at-risk personnel.
5. Personnel enter and exit the laboratory through the clothing change room. When personnel leave the laboratory, they must completely change clothes and shower. The clothes are then decontaminated before being laundered.
6. The BSL-4 laboratory has a dedicated nonrecirculating ventilation system, which is filtered through a HEPA filter before being exhausted.
7. All material is decontaminated before leaving the BSL-4 laboratory.

Examples of BSL-4 agents include Marburg and Congo-Crimean hemorrhagic fever viruses.

Hazardous waste

The clinical laboratory is responsible for the proper handling and disposal of all of the waste it generates. The scope of hazardous waste comprises more than just infectious waste. The *Clinical Laboratory Waste Management Approved Guideline*, 3rd edition, by the Clinical Laboratory and Standards Institute, provides information for laboratory managers on the regulations governing hazardous wastes and addresses the following types of waste: chemical, infectious, radioactive, sharps, multihazardous, and nonhazardous. The guideline also emphasizes methods to reduce waste generation and methods to reduce the volume of and toxicity of unavoidable wastes.

Disposal of infectious waste

In addition to laboratory employees who work with potentially infectious material, the general public must be protected from exposure to these same materials after the laboratory or hospital has disposed of them. The surfacing of contaminated needles and other sharps along lake and ocean beaches led to a public outcry to make hospitals accountable for their infectious waste disposal. In an effort to handle this problem, the U.S. Congress passed the Medical Wastes Tracking Act in 1988 to regulate the states of New York, New Jersey, Connecticut, and Rhode Island as well as Puerto Rico. These regulations went into effect on June 24, 1989, and expired on June 21, 1999. This act allowed the EPA to gather information and focus attention on the problem of medical waste and provided a basis for other states and federal agencies to develop policies on the disposal of medical waste.

The microbiology laboratory's safety program must follow state and local regulations for the safe disposal of its infectious waste, usually by either autoclaving or incineration. Warning signs containing the warning symbol for biohazardous materials must be placed on all biohazard waste and material disposal containers. Figs. 4.5 and 4.6 show disposal containers appropriate for contaminated and biohazardous materials.

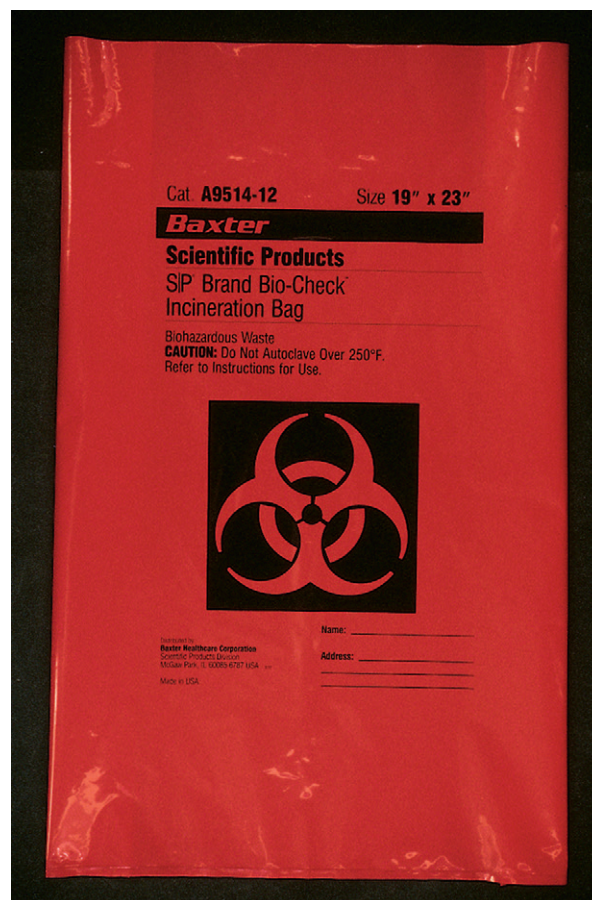


Fig. 4.5 Biohazard bag used to dispose of culture plates and other nonsharp contaminated materials. The bag is sealed and incinerated. The biohazard symbol is in the center of the bag.

Hazardous waste reduction

The EPA has also made recommendations for hazardous waste reduction through the following methods:

1. Substitute less hazardous chemicals when possible.
2. Develop procedures that use less of a hazardous chemical.
3. Recycle chemicals when possible.
4. Segregate infectious wastes from uncontaminated trash.
5. Substitute micromethodology in antimicrobial susceptibility testing and identification of organisms to reduce the volume of chemical reagents as well as infectious waste.

Chemical safety

Chemicals used in the clinical laboratory can be hazardous to the laboratory scientist. The toxic risks of a chemical are related to the extent of exposure and to the inherent toxicity of the chemical. The possible routes of exposure for chemicals are through the skin, eyes, mucosa, gastrointestinal tract, and respiratory tract. The OSHA addresses employee safety with hazardous chemicals in 29 Code of Federal register (CFR) 1910.1200, Hazard Communication Standard (HCS). The HCS focuses on the proper classification of chemicals by manufacturers and importers as well as the safety practices



Fig. 4.6 A and B, Examples of biohazard containers for disposable needles, glass slides, and other sharp materials.

for employers and employees. It requires a laboratory chemical hygiene plan and a hazard communication program, and it states that all clinical laboratory personnel should have a thorough working knowledge of the hazards of the chemicals with which they come into contact, or **employee right-to-know**, and must receive chemical safety training annually. The National Research Council publication *Prudent Practices in the Laboratory: Handling and Disposal of Chemicals* can be used by laboratory managers when developing a chemical hygiene plan for a laboratory. All chemicals in the workplace must be identified and clearly labeled with the **National Fire Protection Association (NFPA) 704 hazard-rating diamond**, stating risk for flammability, reactivity, and health (Fig. 4.7).

Safety data sheets

Safety data sheets (SDSs) are provided by the manufacturer or distributor of hazardous chemicals. Although there is no accepted format regarding what is contained in an SDS, some of the information that should be provided is as follows:

- Name, address, and telephone number of the manufacturer
- Chemical and synonym names and a list of the hazardous ingredients
- Chemical characteristics, such as vapor pressure and flash point, and the physical characteristics (potential for fire, explosion, and reactivity)
- Health hazards of the hazardous chemical (signs and symptoms of exposure and any medical conditions that can be caused by exposure)
- The primary routes of entry and the target organs
- Precautions for safe handling and use, including appropriate hygienic practices

HAZARDOUS MATERIALS CLASSIFICATION CLASIFICACION DE MATERIALES PELIGROSOS

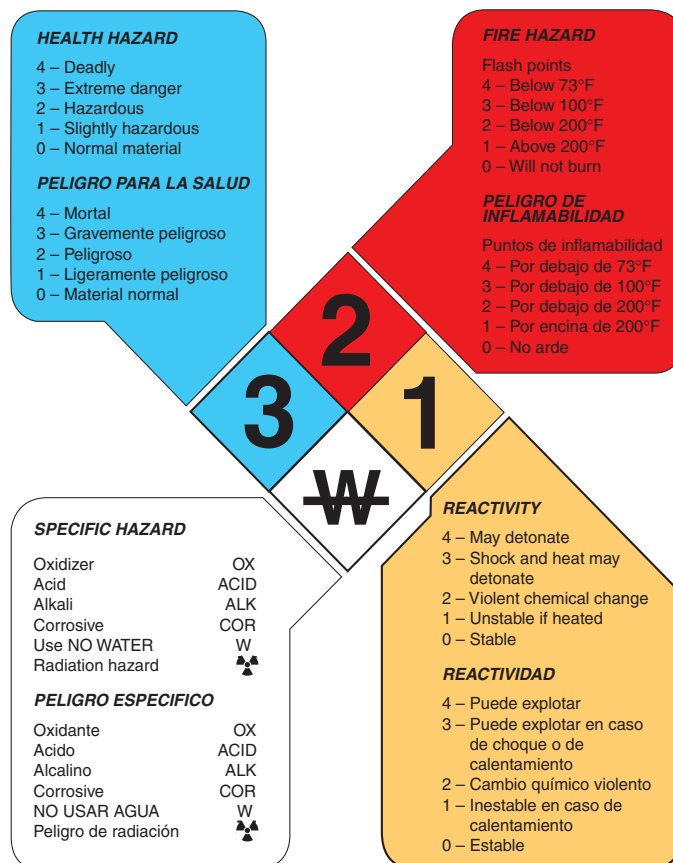


Fig. 4.7 National Fire Protection Association hazardous materials classification symbol. (Courtesy Laboratory Safety Supply, Inc., Janesville, WI.)

- Any generally applicable control measures, such as appropriate engineering controls, work practices, or PPE
- Emergency and first aid procedures
- Spill cleanup procedure
- Disposal recommendations

An example of an SDS is shown in Fig. 4.8. These documents should be kept on file and made available to every employee. Other examples of common hazardous chemicals found in the microbiology laboratory are listed in Box 4.4.



Safety Data Sheet acc. to OSHA HCS

Page 1/9

Date Prepared: 3/25/2021

Reviewed On: 3/25/2021

1 Identification

- **Product Identifier**
- **Product Name:** Streptococcus pneumoniae Antibody-Coated Latex (Pneumoslide Test Kit)
- **Catalog Number:** 240840
- **Relevant identified uses of the substance or mixture and uses advised against**
No further relevant information available.
- **Application of the substance / the mixture** In-vitro Diagnostics
- **Details of the supplier of the safety data sheet**
- **Manufacturer/Supplier:**
BD Diagnostic Systems
7 Loveton Circle
Sparks, MD 21152
Telephone: (410) 771 - 0100 or (800) 638 - 8663
Email Address: Technical_Services@bd.com
- **Information Department:** Technical Service
- **Emergency telephone number:**
In case of a chemical emergency, spill, fire, exposure, or accident, contact BD Diagnostic Systems (410) 771-0100 or (800)-638-8663, or ChemTrec at (800) 424-9300.

2 Hazard(s) identification

- **Classification of the substance or mixture**



GHS07

Skin Sens. 1 H317 May cause an allergic skin reaction.

- **Classification according to Directive 67/548/EEC or Directive 1999/45/EC**
This product contains no hazardous constituents, or the concentration of all chemical constituents are below the regulatory threshold limits described by Occupational Safety Health Administration Hazard Communication Standard 29 CFR 1910.1200, the Canada's Workplace Hazardous Materials Information System (WHMIS) and the European Directive 67/548/EEC and 1999/45/EC.
- **Classification system:**
The classification was made according to the latest editions of international substances lists, and expanded upon from company and literature data.

- **Label elements**

- **GHS label elements**

The product is classified and labeled according to the Globally Harmonized System (GHS).

- **Hazard pictograms**



GHS07

- **Signal word** Warning

(Contd. on page 2)

US

Fig. 4.8 Safety data sheet. (Courtesy and © Becton, Dickinson and Company.)



Safety Data Sheet acc. to OSHA HCS

Page 2/9

Date Prepared: 3/25/2021

Reviewed On: 3/25/2021

**Product Name: Streptococcus pneumoniae Antibody-Coated Latex
(Pneumoslides Test Kit)**

(Contd. of page 1)

· **Hazard-determining components of labeling:**

2-methyl-4-isothiazolin-3-one

· **Hazard statements**

H317 May cause an allergic skin reaction.

· **Precautionary statements**

P101 If medical advice is needed, have product container or label at hand.

P102 Keep out of reach of children.

P103 Read label before use.

P261 Avoid breathing dust/fume/gas/mist/vapours/spray.

P280 Wear protective gloves/protective clothing/eye protection/face protection.

P272 Contaminated work clothing should not be allowed out of the workplace.

P321 Specific treatment (see on this label).

P363 Wash contaminated clothing before reuse.

P333+P313 If skin irritation or rash occurs: Get medical advice/attention.

P302+P352 IF ON SKIN: Wash with plenty of soap and water.

P501 Dispose of contents/container in accordance with local/regional/national/international regulations.

· **NFPA ratings (scale 0-4)**



Health = 0

Flammability = 0

Reactivity = 0

· **HMIS ratings (scale 0-4)**



HEALTH 0 Health = 0

FIRE 0 Flammability = 0

REACTIVITY 0 Reactivity = 0

· **Other hazards**

· **Results of PBT and vPvB assessment**

· **PBT:** Not applicable.

· **vPvB:** Not applicable.

3 Composition/information on ingredients

· **Chemical characterization: Mixture**

· **Description:** Mixture consisting of the following components.

· **Dangerous Components:**

	Natural Rubber Latex		99.88%
CAS: 2682-20-4 EINECS: 220-239-6	2-methyl-4-isothiazolin-3-one	T R24/25; C R34; Xi R43	0.1%

· **Additional information** Risk phrases refer to section 15.

US

(Contd. on page 3)



Safety Data Sheet acc. to OSHA HCS

Page 3/9

Date Prepared: 3/25/2021

Reviewed On: 3/25/2021

**Product Name: Streptococcus pneumoniae Antibody-Coated Latex
(Pneumoslides Test Kit)**

(Contd. of page 2)

4 First-aid measures

- **Description of first aid measures**
- **General information** No special measures required.
- **After inhalation** Seek medical treatment in case of complaints.
- **After skin contact** Immediately wash with water and soap and rinse thoroughly.
- **After eye contact**
Rinse opened eye for several minutes under running water. If symptoms persist, consult a doctor.
- **After swallowing** If symptoms persist consult doctor.
- **Information for doctor** Show this product label or this MSDS.
- **Most important symptoms and effects, both acute and delayed**
No further relevant information available.
- **Indication of any immediate medical attention and special treatment needed**
No further relevant information available.

5 Fire-fighting measures

- **Extinguishing media**
- **Suitable extinguishing agents**
CO₂, ABC multipurpose dry chemical or water spray. Fight larger fires with water spray or alcohol resistant foam.
- **Special hazards arising from the substance or mixture**
No further relevant information available.
- **Advice for firefighters**
- **Protective equipment:** No special measures required.

6 Accidental release measures

- **Personal precautions, protective equipment and emergency procedures** Not required.
- **Environmental precautions:** Wipe up with damp sponge or mop.
- **Methods and material for containment and cleaning up:** No special measures required.
- **Reference to other sections** No dangerous substances are released.

7 Handling and storage

- **Handling**
- **Precautions for safe handling** No special measures required.
- **Information about protection against explosions and fires:**
No special measures required.
- **Conditions for safe storage, including any incompatibilities**
- **Storage**
- **Requirements to be met by storerooms and receptacles:** 2 - 8 °C

(Contd. on page 4)

US



Safety Data Sheet

acc. to OSHA HCS

Page 4/9

Date Prepared: 3/25/2021

Reviewed On: 3/25/2021

Product Name: Streptococcus pneumoniae Antibody-Coated Latex (Pneumoslides Test Kit)

(Contd. of page 3)

- **Information about storage in one common storage facility:**
Store away from oxidizing agents.
- **Further information about storage conditions:**
Store in cool, dry conditions in well sealed containers.
- **Specific end use(s)** No further relevant information available.

8 Exposure controls/personal protection

- **Additional information about design of technical systems:**
No further data; see Section 7.
- **Control parameters**
- **Components with limit values that require monitoring at the workplace:**
The product does not contain any relevant quantities of materials with critical values that have to be monitored at the workplace.
- **Additional information:** The lists that were valid during the creation were used as basis.
- **Exposure controls**
- **Personal Protective Equipment**
- **General protective and hygienic measures**
The usual precautionary measures for handling chemicals should be followed.
- **Breathing equipment:**
In case of brief exposure, use a chemical fume hood or a NIOSH/MSHA-approved respirator.
- **Protection of hands:**



Chemical resistant gloves (i.e. nitrile, or equivalent).

- **Eye protection:** Safety glasses
- **Body protection:** Protective work clothing (lab coat).

9 Physical and chemical properties

- **Information on basic physical and chemical properties**
- **General Information**
- **Appearance:**

Form:	Solid.
Color:	According to product specification
Odor:	Characteristic
- **Change in condition**

Melting point/Melting range:	Not determined
Boiling point/Boiling range:	Not determined
- **Flash point:** Not applicable

(Contd. on page 5)

FIG. 4.8, cont'd



Safety Data Sheet acc. to OSHA HCS

Page 5/9

Date Prepared: 3/25/2021

Reviewed On: 3/25/2021

**Product Name: Streptococcus pneumoniae Antibody-Coated Latex
(Pneumoslides Test Kit)**

(Contd. of page 4)

- **Flammability (solid, gaseous)** Product is not flammable.
- **Danger of explosion:** Product does not present an explosion hazard.
- **Density:** Not determined
- **Solubility in / Miscibility with Water:** Insoluble
- **Solvent content:**
- **Solids content:** 100.0 %
- **Other information** No further relevant information available.

10 Stability and reactivity

- **Reactivity**
- **Chemical stability**
- **Thermal decomposition / conditions to be avoided:**
No decomposition if used according to specifications.
- **Possibility of hazardous reactions** No dangerous reactions known
- **Conditions to avoid** No further relevant information available.
- **Incompatible materials:** No further relevant information available.
- **Hazardous decomposition products:** No dangerous decomposition products known.

11 Toxicological information

- **Information on toxicological effects**
- **Acute toxicity:**
- **Primary irritant effect:**
- **on the skin:** No irritating effect.
- **on the eye:** No irritating effect.
- **Sensitization:** No sensitizing effects known.
- **Additional toxicological information:**
The product is not subject to OSHA classification according to internally approved calculation methods for preparations.
When used and handled according to specifications, the product does not have any harmful effects according to our experience and the information provided to us.
This product or product container contains natural rubber latex which may cause allergic reactions. People with known or suspected allergies to latex should use appropriate safety precautions when handling.
- **Carcinogenic categories**
- **IARC (International Agency for Research on Cancer)**
None of the ingredients is listed.

(Contd. on page 6)

US



Safety Data Sheet acc. to OSHA HCS

Page 6/9

Date Prepared: 3/25/2021

Reviewed On: 3/25/2021

**Product Name: Streptococcus pneumoniae Antibody-Coated Latex
(Pneumoslides Test Kit)**

(Contd. of page 5)

· **NTP (National Toxicology Program)**

None of the ingredients is listed.

12 Ecological information

- **Toxicity**
- **Aquatic toxicity:** No further relevant information available.
- **Persistence and degradability** No further relevant information available.
- **Behavior in environmental systems:**
- **Bioaccumulative potential** No further relevant information available.
- **Mobility in soil** No further relevant information available.
- **Ecotoxicological effects:**
- **Other information:**
The ecological effects have not been thoroughly investigated, but currently none have been identified.
- **Additional ecological information:**
- **General notes:** Generally not hazardous for water.
- **Results of PBT and vPvB assessment**
- **PBT:** Not applicable.
- **vPvB:** Not applicable.
- **Other adverse effects** No further relevant information available.

13 Disposal considerations

- **Waste treatment methods**
- **Recommendation**
Dispose of material in accordance with federal (40 CFR 261.3), state and local requirements.
This product is not considered a RCRA hazardous waste.
Smaller quantities can be disposed of with solid waste.
- **Uncleaned packagings:**
- **Recommendation:** Disposal must be made according to state and federal regulations.
- **Recommended cleansing agent:** Water, if necessary with cleansing agents.

14 Transport information

- | | |
|------------------------------------|------|
| · UN-Number | |
| · DOT, ADR, ADN, IMDG, IATA | Void |
| · UN proper shipping name | |
| · DOT, ADR, ADN, IMDG, IATA | Void |

(Contd. on page 7)

US



Safety Data Sheet acc. to OSHA HCS

Page 7/9

Date Prepared: 3/25/2021

Reviewed On: 3/25/2021

**Product Name: Streptococcus pneumoniae Antibody-Coated Latex
(Pneumoslide Test Kit)**

(Contd. of page 6)

· Transport hazard class(es)	
· DOT, ADR, ADN, IMDG, IATA	
· Class	Void
· Packing group	
· DOT, ADR, IMDG, IATA	Void
· Environmental hazards:	
· Marine pollutant:	No
· Special precautions for user	Not applicable.
· Transport in bulk according to Annex II of MARPOL73/78 and the IBC Code	Not applicable.
· Transport/Additional information:	If a "void" appears in the Hazard Class section for the type of transportation, this indicates the product is not regulated for transportation.
· UN "Model Regulation":	-

15 Regulatory information

· **Safety, health and environmental regulations/legislation specific for the substance or mixture**

· **SARA Section 355 (extremely hazardous substances)**

None of the ingredients is listed.

· **SARA Section 313 (specific toxic chemical listings)**

None of the ingredients is listed.

· **TSCA (Toxic Substances Control Act)**

2682-20-4 2-methyl-4-isothiazolin-3-one

30007-47-7 5-Bromo-5-nitro-1,3-dioxane

· **California Proposition 65 - Chemicals known to cause cancer**

None of the ingredients is listed.

· **California Proposition 65 - Chemicals known to cause reproductive toxicity for females:**

None of the ingredients is listed.

· **California Proposition 65 - Chemicals known to cause reproductive toxicity for males:**

None of the ingredients is listed.

· **California Proposition 65 - Chemicals known to cause developmental toxicity:**

None of the ingredients is listed.

(Contd. on page 8)

US



Safety Data Sheet acc. to OSHA HCS

Page 8/9

Date Prepared: 3/25/2021

Reviewed On: 3/25/2021

**Product Name: Streptococcus pneumoniae Antibody-Coated Latex
(Pneumoslides Test Kit)**

(Contd. of page 7)

- **Carcinogenic categories**

- **TLV (Threshold Limit Value established by ACGIH)**

None of the ingredients is listed.

- **GHS label elements**

The product is classified and labeled according to the Globally Harmonized System (GHS).

- **Hazard pictograms**



GHS07

- **Signal word** Warning

- **Hazard-determining components of labeling:**

2-methyl-4-isothiazolin-3-one

- **Hazard statements**

H317 May cause an allergic skin reaction.

- **Precautionary statements**

P101 If medical advice is needed, have product container or label at hand.

P102 Keep out of reach of children.

P103 Read label before use.

P261 Avoid breathing dust/fume/gas/mist/vapours/spray.

P280 Wear protective gloves/protective clothing/eye protection/face protection.

P272 Contaminated work clothing should not be allowed out of the workplace.

P321 Specific treatment (see on this label).

P363 Wash contaminated clothing before reuse.

P333+P313 If skin irritation or rash occurs: Get medical advice/attention.

P302+P352 IF ON SKIN: Wash with plenty of soap and water.

P501 Dispose of contents/container in accordance with local/regional/national/international regulations.

- **Chemical safety assessment:** A Chemical Safety Assessment has not been carried out.

16 Other information

To the best of our knowledge, the information contained herein is accurate. However, neither Becton, Dickinson and Company or any of its subsidiaries assumes any liabilities whatsoever for the accuracy or completeness of the information contained herein. Final determination of suitability of any material is the sole responsibility of the user. All materials may present unknown hazards and should be used with caution. Although certain hazards are described herein, we can not guarantee that these are the only hazards that exist.

- **Department issuing MSDS:**

Environmental, Health & Safety

Created by Michael J. Spinazzola

- **Contact:** Technical Service Representative

(Contd. on page 9)

US



Safety Data Sheet acc. to OSHA HCS

Page 9/9

Date Prepared: 3/25/2021

Reviewed On: 3/25/2021

**Product Name: Streptococcus pneumoniae Antibody-Coated Latex
(Pneumoslide Test Kit)**

(Contd. of page 8)

• **Date of preparation / last revision** 03/25/2014 / -

• **Abbreviations and acronyms:**

RID: Règlement international concernant le transport des marchandises dangereuses par chemin de fer
(Regulations Concerning the International Transport of Dangerous Goods by Rail)

IATA-DGR: Dangerous Goods Regulations by the "International Air Transport Association" (IATA)

ICAO: International Civil Aviation Organization

ICAO-TI: Technical Instructions by the "International Civil Aviation Organization" (ICAO)

ADR: Accord européen sur le transport des marchandises dangereuses par Route (European Agreement concerning the International Carriage of Dangerous Goods by Road)

IMDG: International Maritime Code for Dangerous Goods

DOT: US Department of Transportation

IATA: International Air Transport Association

ACGIH: American Conference of Governmental Industrial Hygienists

EINECS: European Inventory of Existing Commercial Chemical Substances

ELINCS: European List of Notified Chemical Substances

CAS: Chemical Abstracts Service (division of the American Chemical Society)

NFPA: National Fire Protection Association (USA)

HMS: Hazardous Materials Identification System (USA)

Skin Sens. 1: Sensitisation - Skin, Hazard Category 1

US

FIG. 4.8, cont'd

BOX 4.4 Hazardous chemicals commonly used in the microbiology laboratory

Flammables

- Methanol
- Acetone
- Ethanol

Potential or proven carcinogens

- Aniline (crystal violet) stain
- Auramine-rhodamine stain

Irritants and corrosives

- Hydrogen peroxide
- Acids: HCl, H₂SO₄, acetic acid
- NaOH

Chemical storage

Chemicals should never be stored alphabetically. Chemicals must be stored according to established rules of compatibility to prevent contact between reactive substances. Do not store alkali metals (e.g., sodium, potassium) with carbon dioxide, chlorinated hydrocarbons, or water. Acids and bases should never be stored together. Also, acids such as acetic acid and sulfuric acid should never be stored with oxidizing agents. Halogens are incompatible with ammonia, acetylene, and hydrocarbons. Flammable chemicals should be stored in a flammable storage cabinet. Within the laboratory, chemicals should be stored in amounts reflective of the daily requirements. Bulk stocks should be stored in specially designated areas and not in the laboratory. It is good practice to store corrosives in trays as secondary containment because of the potential for shelves to become damaged.

Chemicals inventory

The laboratory must maintain a current inventory of chemicals, which must be updated annually, along with the SDSs for those particular chemicals. The following four sources should be consulted in preparing an inventory:

1. 29 CFR Part 1910, Subpart Z, Toxic and Hazardous Substances, OSHA
2. National Toxicology Program Annual Report on Carcinogens
3. International Agency for Cancer Research Monographs
4. Manufacturers' SDSs

Hazardous chemical classification

Chemical manufacturers and importers classify chemicals as required by OSHA Standard 1910.1200(d). Chemical manufacturers and importers determine the hazard classes and the category of each chemical by considering the comprehensive range of scientific literature related to a chemical. There is no requirement for manufacturers or importers to perform tests to determine a chemical classification. Chemical manufacturers and importers determine whether a chemical is carcinogenic by referring to the National Toxicology Program and the International Agency

for Research on Cancer annual reports. End-users of chemicals are not required to classify chemicals; however, they may conduct their own classification studies if they deem such procedures appropriate for their facility.

Laboratory safety for hazardous chemicals

Laboratory fume hoods are one of the most important pieces of equipment for protecting workers from exposure to hazardous chemicals. Fume hoods must be provided to prevent inhalation of fumes and should be evaluated at least annually for adequate face velocity (average velocity of the air drawn through the face of the hood) and proper operation. Fume hoods should be vented. PPE, including appropriate gloves, laboratory coats, and eyewear, must be used in the case of a small spill to protect workers from exposure. A dust mask or respirator may be appropriate depending on the material spilled.

Chemical spills

Spillage in the laboratory is unavoidable and unpredictable. Many chemical manufacturers develop charts describing the procedure for managing spills. Acid and base spill kits and flammable spill kits should be kept in areas where such substances are used. Equipment such as protective clothing, scoops and dust pans, forceps for picking up glass, and buckets should be kept in a designated area. In the event of a large spill, the Environmental Health and Safety Department should be called for assistance.

Signage

Signage is important safety equipment. Warning signs and symbols, as shown in Fig. 4.9, must be placed in appropriate locations. Employees must be able to recognize each of these symbols and must be knowledgeable about the danger each indicates and the proper precautions that must be observed.

Fire safety

Bunsen burners and other open-flame burners in most cases have been replaced with other methods or techniques, such as fixing slides with methanol or slide warmers and using disposable loops. Bunsen burners and open-flame burners are among the biggest sources of fire hazards in the clinical microbiology laboratory. Employees must strive not to be careless or negligent in the use of this equipment. Flammable materials should never be opened near a Bunsen burner in use. Open flames should not be left unattended, and the laboratorian should always be sure to turn off the gas when finished. As previously noted, it is important to never place an open flame inside a BSC.

Another hazard associated with Bunsen burners and other open-flame burners is the fuel. Bunsen burners use gas. A leaking gas line may be a source of explosion or may cause illness in employees who work near the leak. The gas hose should be inspected on a regular basis for cracks, holes, pinched points, or other defects, and the hose should be replaced if any defects are found. If a leak is suspected or the appliance is newly connected, leaks can be checked for by lightly spraying the tubing with soapy water. If bubbles appear when the gas

WARNING SIGNS AND SYMBOLS

POTENTIAL DANGER



Flammable



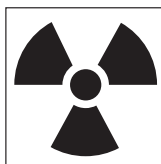
Toxic hazard
Poisonous



Carcinogenic
Cancer-causing agent



Corrosive
Harmful to mucous membranes,
skin, eyes, or tissues



Radiation
Radioactive material present

Fig. 4.9 Miscellaneous warning signs and symbols.

is on, there is a leak at that point. Other burners use a flammable liquid as the fuel, which is in itself a hazard. Other sources of ignition include heating elements, hot plates, incinerators, and spark gaps in motors and light switches.

The OSHA requires employers to have an emergency action plan, which includes what to do in the event of a fire. The emergency action plan must be written, and it must be kept in the workplace and available to all employees. All personnel must be thoroughly trained in the procedure for responding to a fire emergency. Most institutions use the acronym RACE:

- **Rescue:** Remove anyone who is in danger. The safety of handicapped personnel (e.g., deaf, or physically disabled) should be a priority.
- **Alarm:** Know where the nearest fire pull box or alarm station is located and the number to call to report the fire.
- **Contain:** Close doors to contain fire and smoke.
- **Extinguish:** Use the properly rated fire extinguisher on small fires (Table 4.5).

Table 4.5 Classes of fire extinguishers

Symbol	Class	Use
	A	Fires in ordinary combustible materials, such as wood, cloth, paper, rubber, and many plastics; contains water or dry chemical to cool
	B	Fires in flammable liquids, gases, and greases; contains CO ₂ or dry chemical to smother
	C	Fires involving electrical equipment, for which the electrical nonconductivity of the extinguishing media is important; contains CO ₂ or dry chemical to smother without damaging the equipment

If the situation is out of control, the best course of action is to evacuate. The fire evacuation plan must be posted, and employees should be familiar with fire exit locations and evacuation procedures. Periodic fire drills should be conducted to ensure that all personnel react quickly and efficiently in case of a real fire emergency. The drills should include both exit and nonexit procedures. Exit drills familiarize personnel with escape routes and location of fire doors and stairwells. Nonexit procedures alert personnel to the potential for evacuation if the fire is located elsewhere in the building. The laboratory should be kept free from clutter (e.g., do not leave boxes, cords, reagents on the floor), and exits should always remain clear of obstructions.

Thermal injuries

Personnel should be warned of any hot surface or situation in which the potential for burns is present. Use of long thermal gloves that extend to the shoulder is recommended when reaching into autoclaves or hot-air ovens. Signs should be posted warning employees about hot instruments or flasks that have just been sterilized. Burns may also come from extremely low temperatures found with use of liquid nitrogen or freezers that maintain temperatures less than -70°C .

Storage of compressed gases

Flammable and nonflammable gas cylinders in use in the laboratory must always be properly restrained and stored in bulk, and they must be secured in vented areas. In the laboratory, cylinders must be located well away from open flames and other heat sources. Because a leaking pressurized gas cylinder is a potential “missile,” care must be taken to avoid accidental breakage or removal of the pressure valve on top. The metal cap that protects this valve on top of the cylinder must be kept in place during transportation and when the cylinder is not in use.

Electrical safety

Today’s microbiology laboratories contain many instruments. All laboratory electrical equipment must conform to national electrical safety standards and codes. Each instrument must undergo regular preventive maintenance to ensure that it is functioning properly and in the best repair.

Electrical cords should be checked for fraying. All cords should have grounded (three-pronged) plugs. The College of American Pathologists, an organization that provides accreditation to laboratories, requires electrical grounding and leakage checks on instruments before they are put in use, after repairs or modifications, and when there is a suspected problem. Electrical equipment should never be placed near safety showers because of the risk of electrocution.

Miscellaneous safety considerations

Back safety

The best way to care for the back is to prevent back injuries. Carrying heavy trays of culture plates, lifting heavy loads into and out of autoclaves, and sitting or standing improperly all can contribute to back stress or injury. The following are some ways to prevent back injuries:

- Use the legs to lift, not the back.
- Keep loads close to the body when transporting them.
- Ask for assistance or use a cart when a load is too heavy.
- Use good posture.
- Stay physically fit.

First aid training

All personnel should be trained in cardiopulmonary resuscitation and other lifesaving first aid so they will be able to act quickly in an emergency involving either fellow workers or patients.

Immunizations

The OSHA requires that the HBV vaccine be offered free of charge to all personnel who are at risk of exposure to bloodborne pathogens. Microbiology personnel working with sputum specimens or mycobacterial cultures should be screened for exposure to *M. tuberculosis* with an annual tuberculin skin test.

Safety training

All clinical laboratories must offer their employees safety training, and this training must be documented. The training must include the following safety issues:

- *Fire*: Knowledge of how and when to report fires, the location of the nearest alarm box and fire extinguishers, how to use fire extinguishers in small fires, and blocking of fire doors
- *Hazardous materials management*: How to use SDSs
- *Proper storage of gases*
- *Bloodborne pathogens program*: Appropriate practice of infection control, how to handle sharps, compliance with exposure control plan, and handling of biological spills

Safety training programs for microbiology personnel should be conducted annually to keep safe techniques fresh in everyone’s mind. New personnel should be thoroughly trained in safety procedures. It is a good idea to focus on one safety topic at a time and to make the training sessions enjoyable. Safety is each employee’s responsibility.

Bioterrorism and the clinical microbiology laboratory

Clinical microbiology laboratorians are critical players in the early detection of a bioterrorism event. In the September 2001 anthrax incident, laboratorians performed the testing that identified the infectious agent. The CDC has developed a program that identifies how health care workers and other public health officials should respond to bioterrorism event. This program (Emergency Preparedness and Response Program) addresses biological agents and diseases, laboratory information, training, preparedness and planning, and surveillance. Refer to [Chapter 30](#) for more information on agents of bioterrorism.

Laboratory response network

The CDC developed the **Laboratory Response Network** (LRN) in 1999. This program established a network of laboratories that could respond quickly and effectively to biological and chemical terrorism. The LRN developed three levels of laboratories: (1) sentinel laboratories, (2) reference laboratories, and (3) national laboratories. The roles and responsibilities of each of these levels are defined to provide rapid and safe identification of biological agents during a bioterrorism event. The LRN is discussed in [Chapter 30](#).

Sentinel laboratories constitute most hospital-based microbiology laboratories and are divided into two levels. Advanced sentinel clinical laboratories function at the frontlines and have the most capability. The role of these sentinel laboratories is to recognize possible bioterrorism agents and to perform basic testing to rule out these agents or to refer suspicious specimens or isolates to the LRN reference laboratory. Basic sentinel clinical laboratories have fewer analytical capabilities but may handle suspect samples and would refer these specimens to an LRN reference laboratory. The role of reference laboratories and national laboratories in the LRN is to perform confirmatory testing. At the present time, more than 100 laboratories are members of the reference laboratory category. The national laboratories in the LRN are the CDC, the U.S. Army Medical Research Institute of Infectious Diseases, and the Naval Medical Research Center.

Safety during a possible bioterrorism event

The biggest threat with handling agents likely involved in a bioterrorist event is not recognizing the risk associated with these infectious agents and not following the safety procedures instituted in the laboratory for protection. Specimen processing of samples from a possible bioterrorism event should occur within a class II BSC. However, it is likely that the first patients seen in an event will not be identified as victims of bioterrorism. Therefore the cultures from these patients could cause the greatest risk to laboratorians.

The agents that pose the greatest risk are agents transmitted by aerosols, and all laboratorians must remember that

many of the procedures that are performed in the laboratory create aerosols, such as pipetting, flaming loops, streaking plates, and centrifugation. When a bioterrorism agent is suspected, all manipulations of the culture should be performed using BSL-3 guidelines. Sentinel laboratories should *not* accept environmental samples for testing because of the unknown nature of the sample.

Packaging and shipping of infectious substances

Laboratory personnel who are involved in the packaging and shipping of infectious materials must be trained and certified before shipping infectious material. Personnel must be retrained on a regular basis. The International Air Transport Association, the International Civil Aviation Organization, and the U.S. Department of Transportation regulations must be followed when packaging and shipping infectious agents. Laboratory personnel must be aware of what agencies will transport these materials to the identified LRN reference or national laboratory.

POINTS TO REMEMBER

- Physical and chemical methods may be used in the process of sterilization to remove all forms of life.
- Disinfection involves removal of pathogenic organisms but may not include removal of bacterial or other spores; most disinfectants are chemical agents.
- Factors that influence the degree of killing include types of organisms and number of organisms present, concentration of disinfecting agent, amount of soil present, and nature of the surface to be disinfected.
- Antiseptics are designed to reduce the bacterial load of living tissues.
- Disinfectants are designed to be used on inanimate objects to kill or destroy disease-producing microorganisms.
- There are two options a manufacturer can pursue in seeking approval for a disinfectant product: submission of an NDA or OTC drug review known as the *monograph system*.
- Antimicrobial agents for health care personnel use must meet certain standards that demonstrate the product's safety and efficacy.
- Major sources of biological hazards come from patient samples during processing and handling of actively growing culture materials.
- Protective equipment should be used appropriately.
- Safety policies and procedures should be reviewed annually and always followed.
- The microbiology safety program includes proper and safe disposal of infectious waste material.
- OSHA regulations for bloodborne pathogen protection should be followed.
- Chemical and fire safety hazards must be identified, and measures to prevent chemical spills should be employed.
- Continuing education programs to train laboratory personnel in all aspects of laboratory safety and exposure control should be in place.

LEARNING ASSESSMENT QUESTIONS

1. What is the difference between sterilization and disinfection?
2. Explain situations in which you would use a disinfectant and an antiseptic.
3. Describe the difference between physical and chemical methods of disinfection and sterilization.
4. What method is required to kill endospores effectively?
5. List and describe factors that influence the degree of killing during disinfection and sterilization.
6. Explain EPA regulations on chemical surface disinfectants and FDA regulations on chemical skin antiseptics.
7. Transient biota of the skin is defined as:
 - a. Organisms that are contracted from the environment
 - b. Organisms that are contracted from other persons
 - c. Organisms that are not part of the established normal biota
 - d. All of the above
8. Which of the following characteristics should be considered when selecting an antimicrobial agent?
 - a. Spectrum of activity
 - b. Rate of action
 - c. Mechanism of action
 - d. All of the above
9. List the mechanism of action for each type of chemical agent commonly used in antiseptics and disinfectants.
10. Explain the use of health care personnel handwash, surgical hand scrub, and patient preoperative skin preparation.
11. How does the Coronavirus Aid, Relief, and Economic Security Act (CARES Act) reform the OTC drug review?
 - a. Includes important reforms that modernize the way OTC monographs are regulated in the United States
 - b. Replaces the rulemaking process with an administrative order process for issuing, revising, and amending OTC monographs
 - c. Provides the FDA the authority to assess and collect user fees dedicated to OTC monograph drug activities
 - d. All of the above
12. The OSHA requires employers to offer the hepatitis B virus vaccine free of charge to employees who are at risk for exposure to hepatitis B virus. True or False?
13. Safety data sheets contain information relating to which of the following?
 - a. Bloodborne pathogens
 - b. Fume hoods
 - c. Chemical safety
 - d. Fire extinguishers
14. Which of the following would be a correct definition of standard precautions?
 - a. Wearing only gloves to handle blood and body fluids
 - b. Viewing all specimens as potentially infectious and using the appropriate protective equipment
 - c. Delaying testing of blood and body fluids pending results of HIV and hepatitis B antigen testing
 - d. Flagging only specimens that come from patients who are known HIV carriers for "extra precautions"
15. Which type of filter does a class II BSC use to remove infectious agents?
 - a. Millipore filter
 - b. HEPA filter
 - c. Dust filter
 - d. Charcoal filter
16. Infectious agents can enter the body through which of the following routes?
 - a. Inhalation
 - b. Ingestion
 - c. Inoculation
 - d. All of the above
17. Employees can remember the steps to take in case of a fire by remembering which of the following acronyms?
 - a. RUSH
 - b. REST
 - c. RACE
 - d. RISK
18. How often must safety training for laboratory employees be conducted for compliance with OSHA regulations?
 - a. Annually
 - b. Quarterly
 - c. At the time of employment only
 - d. No set requirement
19. Briefly describe the NFPA hazard-rating diamond found on all chemical containers to warn employees of potential hazards associated with that chemical.
20. Washing hands frequently, disinfecting work areas, using needle-resheathing devices, performing procedures in a manner to reduce splashes, and transporting specimens in well-constructed leakproof containers are examples of which of the following?
 - a. Standard precautions
 - b. Work practice controls
 - c. Responsible methods of fire safety
 - d. Chemical hygiene
21. The OSHA Bloodborne Pathogens Standard outlines the safety requirements that an employer must have in place to protect the employee from bloodborne pathogens. This is called:
 - a. Laboratory Response Network
 - b. Work practice controls
 - c. Exposure control plan
 - d. Emergency action plan
22. A patient with active tuberculosis is admitted to the hospital. What type of precautions above and beyond standard precautions will be followed for this patient?
 - a. Airborne precautions
 - b. Contact precautions
 - c. Droplet precautions
 - d. No necessary precautions
23. The following BSL practice is required for agents that may cause serious disease:
 - a. BSL-1
 - b. BSL-2
 - c. BSL-3
 - d. BSL-4
24. The risk group classification for infectious agents that can cause human disease but for which effective treatments and preventive measures are available is:
 - a. Risk group 1
 - b. Risk group 2
 - c. Risk group 3
 - d. Risk group 4
25. OSHA Bloodborne Pathogens Standard requires employers to have an exposure control plan and should include:
 - a. A plan to investigate all exposure incidents and a plan to prevent these from recurring

- b. Method of compliance for standard precautions
- c. Personal protective equipment (PPE)
- d. Guidelines for the handling and disposal of regulated waste
- e. All of the above

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5

Performance improvement in the microbiology laboratory

Connie R. Mahon and Olga Kochar

CHAPTER OUTLINE

Introduction to quality management system, 103

Workflow, 103

Quality and performance improvement measures, 105

Mission, vision, and values, 105

Personnel competency and laboratory proficiency assessments, 105

Continuous education and training, 106

Indicators of performance improvement, 106

Establishing performance monitors, 107

Lean and six sigma in the laboratory, 108

Problem-action form, 108

Learn from defect, 109

The customer concept, 109

Fixing the process, 109

Benchmarking, 109

Quality control in the microbiology laboratory, 110

Temperature, 110

Thermometer calibration, 110

Media quality control, 111

Reagent quality control, 113

Antimicrobial susceptibility quality control, 114

Use of stock cultures, 115

Quality control manual, 115

Individualized quality control plan, 115

Evaluating and interpreting diagnostic laboratory tests, 116

Analytic analysis of tests, 116

Analytic (technical) sensitivity and specificity, 116

Accuracy, 116

Clinical analysis of tests, 116

Clinical (diagnostic) sensitivity, 116

Clinical (diagnostic) specificity, 117

Operational analysis of tests, 117

Incidence of disease, 117

Prevalence of disease, 117

Predictive values of tests, 117

Efficiency of tests, 118

Choosing a laboratory method, 119

Test validation, 119

Bibliography, 121

OBJECTIVES

After reading and studying this chapter, you should be able to:

1. Differentiate quality control (QC) from quality assurance as it applies in the clinical microbiology laboratory.
2. Describe the three phases in a laboratory workflow and what typically occurs in each phase.
3. Discuss the purpose of an organization's mission and vision statements and shared values.
4. Differentiate personnel competency assessment from proficiency testing.
5. Describe the various performance indicators applied in performance improvement.
6. Explain the components of a performance monitor.
7. Justify the benefit of implementing Lean and Six Sigma in the microbiology laboratory.
8. Apply performance monitors such as Problem-Action Form and Learn from Defect.
9. Define benchmarking as it applies to the microbiology laboratory.
10. Establish QC guidelines in a microbiology laboratory including how to monitor equipment maintenance and performance, culture media and reagent performance, personnel competency, use of stock cultures, and the development and updating of procedure manuals.
11. Properly document and institute correction actions.
12. Define individualized QC plan.
13. Explain the significance of customer concept in quality management.
14. Differentiate analytic sensitivity from analytic specificity of laboratory tests.
15. Justify the value of clinical sensitivity and specificity of laboratory tests.
16. Differentiate prevalence from incidence of a disease.

17. Discuss the importance of prevalence in determining the predictive values of laboratory tests.
18. Demonstrate how predictive values of laboratory tests are calculated.
19. Apply the concepts of predictive values of laboratory tests to various clinical examples.
20. Using the formula, calculate the sensitivity and specificity of a particular diagnostic laboratory test.

KEY TERMS

Accuracy	Individualized quality control plan (IQCP)
Analytic activity	Lean
Analytic sensitivity	Learn from Defect
Analytic specificity	Negative predictive value (NPV)
Bayes's theorem	Outcome monitors
Benchmarking	Performance improvement (PI)
Bias	Postanalytic activity
Clinical (diagnostic) sensitivity	Positive predictive value (PPV)
Clinical (diagnostic) specificity	Prenalytic activity
Clinical Laboratory Improvement Amendments (CLIA)	Precision
Clinical and Laboratory Standards Institute (CLSI)	Predictive value
College of American Pathologists (CAP)	Prevalence
Competency	Preventive maintenance
Continuous quality improvement (CQI)	Process monitors
Continuing education (CE)	Proficiency testing
Cross-functional teams	Q-probes
Customer concept	Quality assurance (QA)
Detection limit	Quality control (QC)
Focused monitors	Six Sigma
Incidence	Test validation
	The Joint Commission (TJC)
	Total quality management (TQM)

Case in point

A 5-year-old girl complains of a sore throat. A low-grade fever (99° F [37° C]) is noted when the patient is seen in the physician's office. Her pharynx is red, exudate is present, and her tonsils are swollen. The physician requests a group A streptococcal direct antigen test, and the test is negative. A follow-up culture is positive for group A streptococci 24 hours later.

Issues to consider

After reading the patient's case history, consider:

- All of the intricate processes involved in quality assurance
- How the sensitivity and specificity of a laboratory test is used in the clinical diagnosis of a disease
- How activities at each phase of the workflow (preanalytic, analytic, and postanalytic) affect the accuracy of laboratory testing
- How the prevalence of a disease in a population affects the predictive value of laboratory tests
- Why it is important to constantly validate methods and procedures used in the laboratory

Providing accurate results in laboratory testing is of the utmost importance in clinical settings and public health. The consequences of inaccurate results can be devastating because the patient's health outcomes depend on accurate test results and timely reporting for effective treatment. All phases of laboratory testing must be performed with ultimate precision. Results must be reported to the primary care provider at the time when clinical decisions regarding patient's treatment plan are to be made.

The issue of quality in the laboratory is complex. Because laboratory operations performed by various laboratory personnel involve numerous processes and procedures, a performance management and improvement system must be in place to achieve consistent, quality laboratory performance.

This chapter describes quality management and performance improvement as they apply in the clinical microbiology laboratory. It provides performance monitors and evaluation tools to assure quality performance, guidelines for establishing quality control (QC) measures, and how to document and institute corrective actions. This chapter also describes the value of test validation, clinical applications of test sensitivity, specificity, and predictive values of diagnostic tests.

Introduction to quality management system

Laboratories have always taken measures to ensure that the testing performed on patient specimens produces reliable and accurate laboratory data. This effort has been termed **quality control (QC)**. Examples of QC in the clinical microbiology laboratory are checking media and reagents with specific organisms to determine whether expected results are obtained and documenting that instrumentation meets all operating parameters before it is used to process patient samples.

Over the past few decades, however, concepts and applications of quality management have evolved. Laboratory professionals now recognize that QC is only a small part of the overall assessment of quality within the entirety of the laboratory workflow. **Quality assurance (QA)**, a process that involves monitoring all components of a system or procedure (preanalytical, analytical, and postanalytical) and implementing changes when suboptimal performance is identified, involves taking proactive measures to ensure high-quality patient care.

Even when the laboratory has effectively controlled media, reagents, and instruments, the quality of the test result is poor if the specimen was improperly collected or degraded before arriving in the laboratory and was still assayed. Suppose a specimen is labeled with the wrong patient's name; again, the media can be of top quality, the incubator temperature accurate, and the laboratory scientist very competent, but if the results are reported for the wrong patient, quality patient care management for the patients involved is absent.

Workflow

The goal of a quality management system is continuous performance improvement in laboratory processes. All

processes and procedures must be conducted correctly to ensure that the test results are accurate and reliable. Laboratory testing has become more complex. Performance improvement and quality management systems must address all areas of the laboratory operations that are systematized in a workflow.

Workflow is the series of activities that are necessary to complete a task. In the clinical and laboratory diagnosis of an infectious disease, for example, workflow may begin with the specimen collection at the point-of-care, where the clinician or health care provider sees the patient. Each step in the workflow must be completed with accuracy and precision in as short a time as possible. Workflow in the clinical laboratory, divided into three phases, preanalytical, analytical, and postanalytical,

is critical in managing performance improvement and quality management. Fig. 5.1 shows the three phases of workflow with examples of activities at each phase that can affect testing outcomes. The first phase, *preanalytical*, includes collection of samples at the patient bedside or in the clinician's office. Studies have shown that most errors occur in the preanalytical phase starting with specimen collection, handling, and patient specimen identification. Errors that occur in this phase may eventually continue down the line; hence, large institutions have added the use of technology and robotics as tools in their attempt to lessen missteps in the preanalytical phase.

The actual testing or performance of procedures on clinical samples to produce results occurs in the *analytical phase* of laboratory testing. It is important that the laboratory utilize

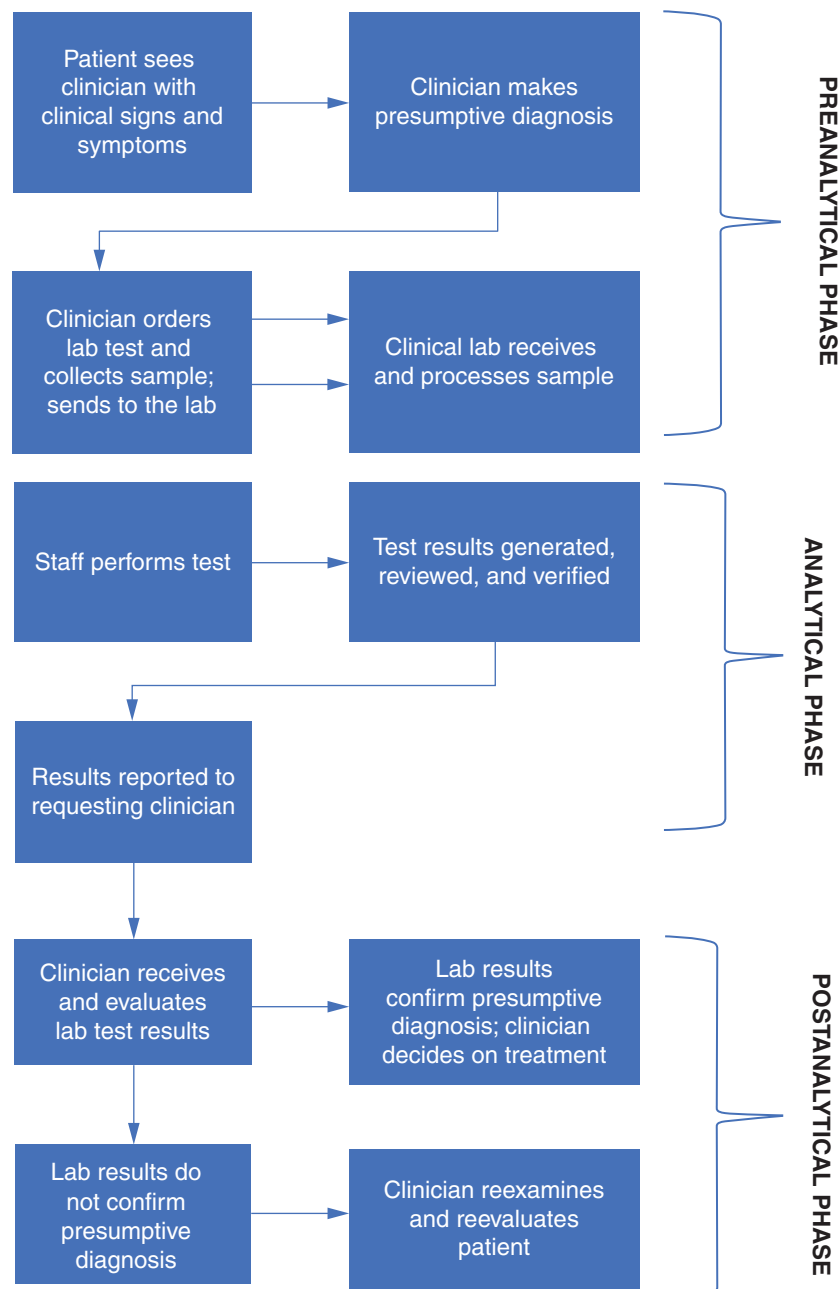


Fig. 5.1 Three phases of laboratory workflow with activities that affect testing outcomes.

all types of diagnostic means that provide rapid and accurate results for the clinician to make a diagnosis or other clinical decisions. The final phase is the *postanalytical* phase in which results generated by an instrument or by a laboratory practitioner (if the test is performed manually) are reviewed, verified by a technical supervisor, and reported to the requestor, typically a health care provider. It is important to realize that *preanalytical*, analytical, and *postanalytical activities* all affect quality. An outcome can be interrupted or compromised at any point in the process.

Case check 5.1

Whereas direct antigen tests for group A streptococci have improved, they are prone to false-negative results. However, specimen collection and processing could also have been performed incorrectly. As mentioned earlier, reports have shown that most errors occur in the preanalytical phase of the workflow such as during specimen collection. Monitoring of false-negative results for direct antigen tests for group A streptococci could identify a pattern requiring intervention to improve results.

The Joint Commission (TJC), the nonprofit organization that accredits health care organizations, focuses on performance measures of organizational systems that are critical to patient safety, care, and treatment. In the ever-changing health care arena, the pursuit of quality continues with concepts that integrate all aspects of health care. These concepts, called **total quality management (TQM)** and **continuous quality improvement (CQI)**, are continuous, incremental improvement processes that reflect the organization-wide philosophy that quality is everybody's responsibility. Because of the complexity of laboratory operations, performance improvement (PI) and quality management (components of TQM and CQI systems) must direct activities toward all areas that include the laboratory environment, personnel competency, QC procedures, quality of testing reagents, equipment, record keeping, and communications, to ensure quality.

Quality and performance improvement measures

Accrediting agencies such as TJC and the College of American Pathologists (CAP) emphasize PI in their accreditation checklists. The Clinical Laboratory Improvement Amendments (CLIA) also require the use of written PI policies in laboratories that include all three phases of testing. Every laboratory must have a well-established Performance Improvement Plan (PIP). To effectively improve quality and sustain reliable and reproducible laboratory performance over time, all employees must understand the entire plan and how it applies to their role within the laboratory and be active participants in the PIP.

Mission, vision, and values

The focus of a PIP is primarily on the processes, procedures, and workflow design, to resolve challenges and ensure continuous delivery of quality work and test results. Although

laboratories operate with processes and test procedures to produce results, understanding other aspects of the organizational design and structure (such as its mission, vision, and values) and their significance is also important in successfully managing and evaluating work processes.

Mission statement

Sharing the organization's mission statement with employees is an effective way to unite everyone behind the same cause. A mission statement expresses the organization's reason for existence. It describes the task, purpose, or calling—what it does and its overall intention. The mission statement supports the vision and serves to provide direction to employees, customers, and other stakeholders. In health care, this includes the patients that the health care institution serves. An example of a mission statement is at Ohio State University Wexner Medical Center: "to improve people's lives in Ohio and across the world through innovation in research, education and patient care."

Vision statement

While a mission statement defines its purpose, a vision statement describes an organization's aspirations or what it hopes to be in the future. The vision, looking into the future, is an image of the mission accomplished. As a guide when developing a vision statement, leaders try to answer the question "What will the organization look like 10 years from now if it were to accomplish all of its strategic goals?" If the mission statement provides direction and purpose to employees, the vision statement should provide inspiration and aspiration. At Mayo Clinic, the vision statement is "Mayo Clinic will provide an unparalleled experience as the most trusted partner for health care."

The laboratory must highlight the organization's vision and mission statements so personnel understand that they each have a role in the mission, and they must strive to make PI part of their daily lives to achieve the organization's vision. They must view challenges as opportunities for improvements and a chance to excel. Employees who find an organization's vision statement meaningful are more engaged, and more engaged employees are more productive.

Shared values

What serve as the moral compass for the organization's practices and its employees, guide their decision-making process, and provide the standards that guide the individual employee's behaviors are the organization's defined values shared by its employees. These core values guide the organization and its employees as they pursue new priorities and are the basis for the organization's vision and mission statements. As an example, Mayo Clinic's value statement states, "The needs of the patient come first."

Personnel competency and laboratory proficiency assessments

Competency, the ability of an individual to perform a task accurately and effectively, has played a major role in quality

management and performance improvement in the clinical laboratory. Hence, identifying the competencies for each job function is crucial and has an enormous impact in personnel competency assessment and in implementing work quality and performance improvement.

Personnel competency assessment

The CLIA (the first one was passed as the Clinical Laboratory Improvement Act of 1988 [CLIA-88]) requires that the competency of each employee be determined and verified on employment. Proof of competency must be maintained in each employee's personnel file. Competency reverification must occur annually. This requirement is to ensure that laboratory professionals are meeting the standards in test performance as required by federal regulation in order to avoid errors that could lead to harming patients. Competency assessment is different from proficiency testing under CLIA. While **competency assessment** determines the ability of laboratory personnel to perform their duties satisfactorily, **proficiency testing** (PT) assesses the ability of the laboratory to produce accurate and reliable test results by providing them with unknown samples prepared by a Centers for Medicare and Medicaid Services (CMS)-approved PT program. Proficiency testing, as it pertains to the laboratory, is discussed later in this section.

The clinical laboratory is one of the most highly regulated workplaces in health care. Laboratory personnel must meet strict quality standards, from specimen processing to assess acceptability to reporting of results. CLIA requirements expect that laboratories assign a complexity rating to all tests or analyses performed by the laboratory. In microbiology, all tests are moderately complex or highly complex. Microbiology laboratory personnel must meet certain educational requirements before they are allowed to perform at each level of complexity. Personnel competency at each level of complexity testing must be assessed and documented before they are cleared to perform tests on patients' samples independently. Competency assessment of testing personnel is required by CLIA to be completed at least semiannually during the first year the laboratorian performs tests on patient specimens and then annually thereafter. According to CLIA, an individual designated as a Technical Consultant for moderate complexity testing and the Technical Supervisor for high complexity testing are responsible for developing and administering competency assessments to personnel to ensure that they have the proficiency level required to perform laboratory testing.

A common method to administer competency testing is to give laboratory personnel samples as unknowns. Competency is determined by use of a variety of techniques, such as the following:

- Direct observations of routine patient test performance including patient preparation, if applicable, specimen handling, processing, testing; monitoring the recording and reporting of test results
- Review of worksheets
- Assessment of test performance through testing previously analyzed specimens, internal blind testing samples, or external proficiency testing samples
- Assessment of problem-solving skills with written examination

An example of a competency check-off form is shown in Fig. 5.2. Personnel competency must be assessed twice a year on all tests performed on patient samples. Proficiency samples for demonstrating competency may be purchased commercially or prepared internally. The periodic assessment of employee's proficiency on competencies identified for the employee's job role or function provides information on possible skill gaps; assists in a focused, effective training and development plan; and guides both employee and manager in the development of learning plans to help the employee fill the skill gaps.

Proficiency testing

TJC and the CLIA require that laboratories enroll in a CMS-approved proficiency testing program for each regulated analyte tested. Laboratories are required to maintain successful performance on proficiency testing. PT is an example of an external quality management assessment and allows laboratories to compare their performance to the performance of other similar laboratories participating in the same PT event (interlaboratory comparison). Unsuccessful performance is defined as a failure to achieve satisfactory performance for two consecutive or two of three consecutive testing events. When unsuccessful PT occurs, TJC will request a plan of action. If the problem cannot be resolved, an onsite evaluation may be conducted, which can affect accreditation status and force the laboratory to stop testing.

PT samples are available from several approved providers, and a list is available at the CMS website. PT samples must be assayed in the same manner as patient material, except that no PT sample shall be referred to another laboratory for analysis. A laboratory must not test PT samples on more than one instrument or by multiple methods unless that is how patient specimens are processed. In microbiology, microorganisms in proficiency tests must be identified in the same manner as clinical specimens.

Continuous education and training

A quality management and performance improvement plan may also include ongoing professional development, continuous education, and training requirements for all personnel. These **continuing education** (CE) programs may include didactic lectures on new nomenclature, emerging pathogens, or newly developed techniques. Case study presentations or training on new instrumentation are other approaches to CE of laboratory personnel. It is essential that CE programs attended by personnel are documented. Training on new instrumentation should also be documented in the individual employee's personnel competency file.

Indicators of performance improvement

Many types of monitors or indicators can be incorporated into a performance improvement program. Accrediting agencies generally make sure that a variety of types are used. Some monitors are ongoing data collections even when no problems are expected. Results are compiled and routinely evaluated. This procedure establishes a trend and makes problems easy to detect as disruptions in the trend. These are often referred to as **process monitors**.

Employee name: Carol Johnson Year 2020

Demonstrates following abilities:	Work station		
	#1 Respiratory	#2 Urine	#3 O & Ps
1. Handles specimens safely during testing, storing, and discarding	✓	✓	✓
2. Prepares specimens for analysis according to laboratory policies and procedures (parasitology and special procedure areas)	—	—	✓
3. Analyzes specimens according to laboratory procedures for the workstation; knows theory and principles of the tests being performed	✓	✓	✓
4. Clearly records all work done so that another person could take over the workstation	✓	✓	✓
5. Reports results accurately and in a timely manner	✓	✓	✓
6. Makes appropriate critical value and courtesy calls	✓	✓	✓
7. Consistently performs and records quality control and documents all remedial action	✓	✓	✓
8. Remedial action necessary (yes or no)	No	No	No

Remedial actions: _____

Date remediation completed: _____

Evaluator signature A Holbrook Date 2/11/20 (Workstation 1)

Evaluator signature L Creme Date 6/9/20 (Workstation 2)

Evaluator signature S Young Date 8/27/20 (Workstation 3)

Employee signature Carol Johnson Date 8/28/20

Fig. 5.2 Competency documentation.

Outcome monitors are measurements of the result of a process, such as complications that a patient experiences as the result of a process. Other monitors may be created in response to a suspected problem. Data may be collected for a short period to resolve a specific issue. These monitors are called **focused monitors**.

Establishing performance monitors

As an accrediting agency, TJC makes recommendations for establishing performance monitors. The recommended components for a performance monitor are to plan, design, measure, assess, and improve.

BOX 5.1 Measurable processes

Patient preparation
 Specimen handling
 Collection
 Labeling
 Preservation
 Transportation
 Communication processes
 Transfer of information
 Completeness of testing request
 Reporting timeliness
 Report accuracy
 Test appropriateness
 Patient needs and expectations
 Risk management activities
 Quality control activities

Plan: The plan is not expected to be a single-department approach. Rather, it should be a coordinated, organization-wide approach for improving patient outcomes that includes interdisciplinary collaborative actions. The plan phase is about developing an “aim statement” for the project to help stay focused and motivated. The aim statement should be precise and not too broad to ensure overall success of the project.

Design: Clear objectives are needed to describe a new process, component, or service. During this phase of the project, it is important to evaluate the “domino effect” and consider which stakeholders these potential changes can affect so that multidisciplinary discussions can take place if need be, and processes, policies, and procedures can be modified as needed.

Measure: Systematic data collection is necessary for improvements or for ongoing measurements. [Box 5.1](#) lists suggested measurable processes.

Assess: A review of the collected data should be systematic, interdisciplinary, and interdepartmental as well as statistically based using analytic tools. Internal comparisons or comparisons with similar processes in other organizations are appropriate. Guidelines for assessments might be accreditation standards, practice guidelines, or legal and regulatory requirements.

Improve: Current processes or a design of new processes may need to be redesigned. Because more opportunities are usually identified than can be acted on, priorities must be established. Priorities are often based on risk, frequency, or high-volume processes. Some of the good ideas that a team identified along the way, but are not relevant to the particular project, may be moved into the “parking lot” and saved for future projects. It is important to stay focused and disciplined so that projects are documented, changes are implemented, and results are achieved systematically and reliably.

Lean and Six Sigma in the laboratory

First developed by the Japanese automotive industry, the central idea of Lean is to minimize waste while at the same time maximizing customer value. In implementing Lean principles, the laboratory is able to create efficient processes and eliminate wastes as identified by customers.

Examples include waste of motion, materials, effort, and time. In the laboratory, standardization of work processes is a Lean opportunity. It ensures all personnel handle tasks the same way. When standard operating procedures (SOPs) are in place, errors are minimized and ultimately, patient care improves. Another example of how Lean is applied in the laboratory is in the design of space that is centered either around an activity or around a type of specimen. Such Lean design saves time and improves workflow. In the microbiology laboratory, consider where there is waste of motion or time, especially in specimen collection and transport of infectious materials.

The underlying principle of **Six Sigma (6σ)**, another tool for process improvement, is that any deviation in a process presents opportunities for errors, which then leads to product defects and customer dissatisfaction. By applying Six Sigma methodology, causes of deficiencies in the process may be identified and chances for errors can be reduced. Six Sigma (the Greek letter *sigma* is used to denote variation from standard) uses statistical methods. The organization may form an infrastructure of people who are experts in these methods.

What does a *Sigma* value mean? Simply explained, a *Sigma* value or *Sigma* level designates how often mistakes are likely to occur in a process or procedure and represents the number of errors per 1 million opportunities—the higher the Sigma, the fewer the number of errors. In the clinical laboratory, a process to be considered high quality will have to make a Sigma score of six, which is the highest level that can be achieved. That means to achieve Six Sigma, the process must not produce more than 3.4 failed QC results per 1 million. When Six Sigma methodology is applied across the laboratory workflow: preanalytical, analytical, and postanalytical. It can assist with improving accuracy and strengthening the laboratory’s QC activities.

Because medical providers rely heavily on accurate laboratory test results to confirm or reject a diagnosis, initiate therapy, or make other clinical decisions on patients, reducing the chances of errors in a laboratory test result is crucial in saving patients’ lives. Implementing quality management and process monitors like Lean and Six Sigma is appropriate in creating an environment supportive to process evaluation and performance improvement.

Problem-action form

Another approach to monitoring or documenting quality issues is a problem-action form. This approach is commonly used to document issues that are quickly resolved but could also be used for long-term monitor summation. The form is a brief statement consisting of the following information:

- Date
- Problem
- Evaluation and investigation
- Corrective action
- Outcome

The form may be signed by the person submitting it, and additional documentation may be attached as necessary. [Table 5.1](#) illustrates the use of the problem-action form.

Table 5.1 Clinical microbiology problem-action report

Date	11/15/21
Problem	A blood culture was required from a patient at the urgent care facility, but no staff member was trained to draw blood cultures. The patient had to drive from the urgent care facility to the hospital to have the blood drawn by a phlebotomist who was trained in the appropriate techniques
Evaluation	No employees at the urgent care facility have been trained to collect blood cultures. The same is true of the clinic outpatient laboratory. This is the second occurrence in about 2 months
Corrective action	Seriously ill patients should not have to travel from one facility to another to have their blood drawn. Laboratory administration was informed of this situation
Outcome	All outpatient sites will receive training and written instructions for the proper collection of blood for culture. Patients will no longer have to drive to the hospital for this service if they are already at an outpatient facility
Submitted by	M. Rausch
Attached documentation?	No

Learn from defect

More recently, another tool, *Learn from Defect*, is being used widely to evaluate risks and eliminate them as much as possible to prevent a similar event from affecting other patients in the future. The tool intends to provide a methodical and structured approach to ensure that every component of the process is assessed, and nothing is left to chance. When *Learn from Defect* is applied in a performance improvement plan, a cross-functional team is tasked in the risk assessment and development of a corrective plan. With *Learn from Defect*, the cross-functional team will do the following:

1. Provide a concise, objective, factual description of the event in question.
2. Carefully review all of the factors that may have contributed to the event. Then, determine which factors contributed negatively to the event and which factors contributed positively to the event. Negative factors are those that potentially may bring harm to the patient; positive factors limit such harm. Rate the most important factors that contributed to the event.
3. Outline how to limit and eliminate the negative factors. Decide what will be done and who will lead the interventions to eliminate those factors.
4. Determine how to evaluate the new process to ensure that it is working as designed, risk is reduced, and the process is sustained.

The customer concept

Like any business, laboratories must focus on the **customer concept**. Who are the customers, and what is a customer's perception of quality? Patients are not the only customers.

Anyone who looks to the laboratory for a service is a customer. Physicians, nurses, insurers, and patients are all customers. Each customer may view quality differently and may have different expectations. Laboratory professionals may judge quality in terms of accuracy, a physician may view it as turnaround time, a patient may view it as compassion and relief from pain, and an insurance company may view it as cost-effectiveness. Customer satisfaction must be surveyed to determine perceptions.

Fixing the process

When patient outcome is less than desirable, the process must be evaluated and corrected. The focus is on the process, not on an individual. The primary rule to follow is to refrain from finger pointing or fault finding. Preanalytic and postanalytic activities usually take place outside the laboratory and require **cross-functional teams** to evaluate and correct the process. Department representatives can easily become defensive and territorial. Barriers to cooperation must be removed, and the easiest way to achieve it is to remain collegial, professional, and patient-centric at all times.

Cross-functional teams must include a trained *facilitator*. Facilitators are most effective when they have no vested interest in the process being evaluated. The facilitator's role on the team is to use problem-solving training and experience to help the team brainstorm and stay on track. In the formation of a cross-functional team, those who are actually doing the work must be represented; the team should not consist solely of supervisory personnel. If the issue is transportation of specimens, for example, the team should include, at minimum, a transporter, a specimen processor, a staff nurse, a medical student or physician representative, appropriate supervisory personnel, and a facilitator. The most accurate and meaningful brainstorming ideas usually come from the people who perform the tasks, not from those who designed the process. When is a process fixed? It is not fixed just because the reason something went wrong is explained. It is fixed only when the problem is prevented from happening again and the new process is reliably sustained.

Benchmarking

A benchmark is a reference point. **Benchmarking** is seeking an industry's or profession's best practices to imitate them and improve. Benchmarking was initially practiced in business and industry but has now become an important part of a hospital quality management program. It is done willingly and openly. There are two main types of benchmarking: business or operations benchmarking and quality benchmarking.

Operations benchmarking

A hospital can join a large group of other hospitals that all share operating statistics. Productivity and cost-effectiveness are two large categories commonly used in a benchmarking comparison. The best performers in a group of hospitals are highlighted. Hospitals can individually and anonymously see where they are by comparison with other hospitals' statistics and then contact the best performers to evaluate their differences. A code of conduct that includes ethics and etiquette is followed when

benchmarking is performed. Although hospitals generally benchmark other hospitals, they often incorporate lessons learned from other successful industries.

Quality benchmarking

Examples of quality benchmarking are commercially purchased monitors. The CAP created a national PI assessment program called *Q-probes*. Laboratories throughout the country subscribe to an annual series of monitors. At least one of these monitors each year has a microbiology focus. *Q-probes* have covered topics such as adequacy of sputum cultures, turnaround time for spinal fluid Gram-stain results, blood culture contamination rates, and appropriateness of ordering patterns for stool specimens. The method of data collection is precisely outlined, and all worksheets and data forms are provided. Information is returned to subscribers in a manner that enables institutions to perform benchmarking.

Quality control in the microbiology laboratory

QC is a small but important part of TQM and CQI, and as such, it contributes to the organizational goal of providing quality patient care. All QC activities that take place must be recorded to prove their existence and create a traceable record that can be used in case troubleshooting or investigation is required. All record sheets must list tolerance limits, when applicable, so the person recording the results will know whether the value being recorded is acceptable. Corrective action must also be recorded when any measurement falls outside a tolerance limit. The responsibility for QC may rest mainly with one person, but in reality everyone must participate if a program is to be successful. A QC program for a microbiology laboratory must include procedures for control of temperature, equipment, media, reagents, and antimicrobial susceptibility testing.

Temperature

Daily temperature checks are required on all temperature-dependent equipment:

- Incubators
- Heating blocks
- Water baths
- Refrigerators
- Freezers

Incubator and refrigerator thermometers are easier to read if they are permanently immersed in glycerol. This helps prevent temperature fluctuations when the door is opened to read the thermometer. Before use, each thermometer must be checked against a reference thermometer from the National Institute of Standards and Technology (NIST), formerly the National Bureau of Standards (NBS). The most efficient method is to check a large batch of thermometers at the same time and at the temperature ranges likely to be used. A common practice in clinical microbiology is to test all thermometers at -20°C , 2°C to 8°C , 37°C , and 56°C . The NIST

thermometer comes with certification papers that list correction factors to be used in various temperature ranges. These correction factors are applied to all values obtained with individual laboratory thermometers. Laboratories arbitrarily determine the acceptable temperature variance. For most routine work, thermometers that differ by 1°C or more from the reference thermometer are discarded.

Thermometer calibration

Thermometers are calibrated by batch on arrival in the laboratory. Procedure 23 in Appendix D on Evolve outlines the calibration procedure. Once the thermometer passes calibration and is placed in use, repeated calibration of the thermometer should not be necessary. Alternatively, thermometers already checked against an NIST thermometer can be purchased. Certificates of calibration should be kept for the life of the thermometer or until the expiration date on the certificate; after that date, the thermometer can be recalibrated or discarded. Thermometers should be checked daily to ensure that gas bubbles have not been introduced into the liquid, making reading the temperature difficult. Gas bubbles can be eliminated by centrifugation or by placing the thermometer at a high or low temperature.

Equipment maintenance

Equipment used in the clinical microbiology laboratory must be tested for proper performance at intervals appropriate for each item or per the manufacturer's recommendation. This process may involve checking the percentage of CO_2 in an incubator daily or measuring the revolutions per minute of a centrifuge twice a year. How often the instrument or equipment needs to be calibrated must be determined and is based on its stability or the manufacturer's recommendation. In newly installed equipment, a system for recording the use of parts and supplies, performance verification, frequency for calibration, and proper operation must be developed. [Table 5.2](#) gives examples of some laboratory equipment, the type of testing done, and the frequency of testing.

Table 5.2 Frequency of equipment testing

Equipment	Test type	Frequency
Incubator	Temperature, CO_2	Daily
GasPak jar	Anaerobiosis, catalyst-heated	Each use
Anaerobe chamber	Anaerobiosis, humidity, temperature	Daily
Biological safety cabinet	Air flow (done by specialist)	Annually or any time hoods are moved
Centrifuge	Check rpm	Every 6 months
Microscope	Cleaned and adjusted	Four times per year or as needed
Autoclave	Temperature Spore testing	Each load Weekly
Balance	Accuracy of weights	Annually

rpm, Revolutions per minute.

A **preventive maintenance** program must be established as an additional control measure. Scheduled maintenance, which may include daily, weekly, and monthly maintenance activities, must be instituted and assigned to personnel responsible for the maintenance program. Preventive maintenance performed on equipment generally involves tasks such as oiling and cleaning, replacing filters, and recalibrating instruments. Keeping an instrument in best condition and functioning at the proper level will extend its lifetime. Fig. 5.3 shows an example of a preventive maintenance log sheet.

Media quality control

Each batch of user-prepared media must be tested for sterility and performance as part of media QC. Documentation must show that the media support the growth of appropriate microorganisms and, if appropriate, inhibit growth of specific microorganisms or produce the correct biochemical response. Records must be maintained for 2 years. The criteria

are established by the **Clinical and Laboratory Standards Institute (CLSI)** and are listed in document M22-A2.

QC for user-prepared medium includes the following:

- All user-prepared media must be tested before use.
- Each medium must be tested with organisms expected to grow or give a positive reaction as well as with organisms expected not to grow or to produce a negative reaction.
- The medium should be tested for sterility and pH.
- The organisms selected for QC should represent the most fastidious organisms for which the medium was designed.
- Testing techniques should be different for primary plating media than those for biochemical or subculture media. Primary plating media should be tested with dilute suspensions of organisms, whereas biochemical media can be tested with undiluted organisms.
- QC testing should be performed according to CLSI recommendations.
- Expiration dates must be established.

MEDIA, REAGENTS, AND SMALL EQUIPMENT	JANUARY	FEBRUARY	MARCH
MEDIA, REAGENTS:			
1. Check refrigerators and freezers for outdated material.	1/21/20 AF	2/24/20 LH	3/28/20 DS
2. Check for "received" and "opened" dating.	1/21/20 AF	2/24/20 LH	3/28/20 DS
THERMOMETERS: Calibrate by batch on arrival.			
PIPETTORS: Calibrate.			3/10/20 MS
pH METER: (Rooms 337 and 354)			
1. Clean the exterior.	1/21/20 AF	2/24/20 LH	3/28/20 DS
2. Replace water in the electrode holder.	1/21/20 AF	2/24/20 LH	3/28/20 DS
3. Check the AgCl level of the electrode.	1/21/20 AF	2/24/20 LH	3/28/20 DS
EYEWASHES: Flush.			
(Rooms 332, 337, 339, 342, 351[2], 354)	1/21/20 AF	2/24/20 LH	3/28/20 DS
STAINING SINK: Clean.	1/20/20 AF	2/24/20 LH	3/28/20 DS
GROUNDING: Check annually.			
REVIEWED BY: M Rausch Page 1 of 5			
YEAR: 2020			
Preventive maintenance is to be performed in the months highlighted for each item listed.			

Fig. 5.3 Preventive maintenance log sheet.

Fig. 5.4 shows a log sheet used for testing media prepared in-house.

Commercially prepared media are tested by the manufacturer. The laboratory must obtain a statement of QC from the manufacturer for all media that the laboratory will not retest. This certificate must be retained for as long as the

laboratory uses the specified media. Nevertheless, certain types of media must be retested by the user. Examples of media that require retesting are chocolate agar, selective media for pathogenic *Neisseria*, and *Campylobacter* media. Fig. 5.5 lists specialty commercially prepared media that have been retested by the laboratory. A list of all media

DATE	MEDIA AND LOT NUMBER	ORGANISM PLATED	QC PLATED DATE	QC READ DATE	QC PASSED/FAILED	INITIALS
3/8/20	TMA	<i>S. aureus</i> <i>S. epidermidis</i>	3/9/20	3/10/20	Pass	MR
3/8/20	Beta toxin	<i>S. agalactiae</i> ⊕ <i>S. pyogenes</i> ⊖	3/9/20	3/9/20	Pass	MR
3/8/20	PSE	<i>E. faecalis</i>	3/9/20	3/10/20	Pass	MR
3/8/20	TSI	<i>P. aeruginosa</i> <i>C. freundii</i>	3/9/20	3/10/20	Pass	MR

Fig. 5.4 Log sheet for testing media prepared in-house. QC, Quality control.

MEDIUM TESTED	MFG	LOT NUMBER	EXP. DATE	STERILITY	TEST ORGANISMS	RESULT	ACTION TAKEN	DATE	TECH.
Chocolate II	BD	L3RTPO	5/23/20	OK	<i>H. influenzae</i> <i>N. meningitidis</i>	Pass	None	3/8/20	MR
GC-Lect	BD	—							
Jembec-Neiss.	BD	A 3NENC	4/3/20	OK	<i>N. gonorrhoeae</i> <i>N. meningitidis</i> <i>S. epidermidis</i> <i>C. albicans</i> <i>E. coli</i>	Pass	None	3/8/20	MR
Campy bld	BD	LINDHK	4/28/20	OK	<i>C. jejuni</i> <i>E. coli</i>	Pass	None	3/8/20	LM
Campy thio	BD	H4E0AJ	2/1/20	OK	<i>C. jejuni</i> <i>E. coli</i>	Pass	None	3/8/20	LM

Reviewed by: _____ Date: _____

Fig. 5.5 Commercial media that have been retested by the laboratory. The result is pass or fail.

requiring retesting can be found in CLSI document M22-A2. In addition, the laboratory must examine all commercially prepared media for the following:

- Moisture: Plates should be free from moisture before use but should never show signs of drying around the edges.
- Sterility: Plates should be free from contaminants.
- Breakage: Petri dishes should not be cracked or broken.
- Appearance: Blood-based plates should not show signs of hemolysis, and any other plate that deviates from the normal color should not be used. Any deterioration should be reported to the manufacturer.

Results of media observations must be recorded along with the lot numbers. Fig. 5.6 shows an example of a media observation log, which helps ensure that good-quality media are used on all patient samples. Corrective action must be taken when a medium does not meet standards. This can be documented on a separate record known as a *media corrective action log* (Table 5.3).

Reagent quality control

With few exceptions, reagents should be tested on each day of use with both positive and negative controls. Reagents that are documented to have consistent and dependable results may be tested less frequently. Some reagents may be tested more than once a day. Reagents that are opened and used

repeatedly should be checked daily for sterility. Examine and follow the manufacturer's package insert for recommended QC requirements.

QC should be performed and documented on all staining procedures at least daily. For the Gram stain, known organisms from the American Type Culture Collection (ATCC) include gram-positive bacteria (*Staphylococcus aureus* ATCC 25923) and gram-negative bacteria (*Escherichia coli* ATCC 25922). All staining procedures must be verified before patient results can be released. Some examples of reagents that should undergo QC in microbiology are as follows:

- All stains
- β -Lactamase
- Catalase
- Coagulase
- Germ tube solution
- Hippurate
- Kovacs reagent
- Nitrate
- Optochin
- Oxidase
- L-Pyroglutamyl- β -naphthylamine
- Typing sera
- Voges-Proskauer broth
- X and V strips

Fig. 5.7 shows a variety of testing methods that might be performed daily at an individual workstation.

DATE	MEDIUM	LOT NUMBER	MOISTURE	STERILITY	APPEARANCE	BREAKAGE	INITIALS
3/4/20	BAP	A2RUWO	✓	✓	✓	✓	MR
3/4/20	MAC	A4RUUH	✓	✓	✓	✓	MR
3/4/20	CHOC	A4RUUX	✓	✓	✓	✓	MR
3/4/20	CNA	A1RUWB	✓	✓	✓	✓	MR
3/4/20	HE	AZRCUF	✓	✓	✓	✓	MR
3/4/20	CIN	AZNEJE	✓	✓	✓	✓	MR
3/4/20	PD	OSU PREP: 1/6/20	✓	✓	✓	✓	MR
3/4/20	GC-LECT	K3RTNZ	✓	✓	✓	✓	MR
3/4/20	SCH	A4NETG	✓	✓	✓	✓	MR
3/4/20	SCH-GV	A3NENN	✓	✓	✓	✓	MR
3/4/20	CDC ANA	AZRWAU	✓	✓	✓	✓	MR
3/4/20	SMAC	OSU PREP: 2/15/20	✓	✓	✓	✓	MR

Fig. 5.6 Media observation. A check in each column indicates that the medium is acceptable.

Table 5.3 Corrective action log of microbiology

Media: chocolate agar lot #:112451 expiration date: 7/6/2021

DATE	TECH INITIALS	DESCRIBE THE MEDIA QUALITY CONTROL FAILURE	CORRECTIVE ACTION 1. QC repeated using current in-stock ATCC 2. QC repeated using newly ordered ATCC 3. New Media lot # used 4. Correct media incubation time 5. Media not exposed to light (MRSA)	QUALITY CONTROLS ATCC #	EXPECTED RESULTS Growth: G No Growth: NG Partial Growth: PG	RESULTS ACCEPTABLE Yes: Y No: N	Reviewed Date/Initials
6/1/2021	MC	No growth seen/observed on QC <i>N. gonorrhoeae</i> ATCC 13609	1. QC repeated using current in-stock ATCC	<i>N. gonorrhoeae</i> ATCC 13609 <i>H. influenzae</i> ATCC 10211 Sterility Check	G G NG	Y Y Y	OK 6/3/2021
ATCC, American Type Culture Collection; MRSA, methicillin-resistant <i>Staphylococcus aureus</i> . MediaQCmc.							

DATE INITIALS

BLOOD CULTURE WORKSTATION QUALITY CONTROL

Month March
Year 2020

		JAR Anaerobic	HEAT CATALYST	CHANGE DESICCANT Date (weekly)	ACRIDINE ORANGE Pos Neg	NaDesoxy/WELLCOGEN Pos Neg	HEATING BLOCKS (35°–37° C) #20 #17
1	LM	OK	✓		N D	+ –	35° 36°
2	AD	OK	✓		N D	+ –	35° 36°
3	AD	OK x 2	✓		N D	N D	35° 36°
4	MCI	OK	✓		+ –	N D	36° 36°
5	MCI	OK	✓		N D	N D	35° 36°
6	LH	OK x 2	✓		N D	N D	35° 36°
7	AD	OK x 2	✓	✓	N D	+ –	36° 36°
8	AD	OK x 2	✓		N D	+ –	36° 37°
9	LH	OK	✓		N D	+ –	36° 36°
10	AD	OK	✓		N D	N D	36° 36°
11	CI	OK	✓		+ –	N D	36° 36°
12	MG	OK	✓		+ –	+ –	36° 36°
13	DS	OK x 2	✓		N D	N D	36° 36°
14	DS	OK	✓	✓	N D	N D	36° 35°

REVIEWED BY: MBT

DATE: 4/4/20

Fig. 5.7 Various testing methods that can be performed daily at an individual workstation. Shown is an example of a 2-week workstation quality control. ND, Not done.

Antimicrobial susceptibility quality control

The CLSI provides guidelines for QC of susceptibility testing. The recommended control organisms are specific strains from the ATCC (Table 5.4). In addition to the organisms listed in Table 5.4, fastidious organisms such as

Haemophilus influenzae and *Neisseria gonorrhoeae* are tested to ensure that the best possible results are obtained when the same type of isolates recovered from patient samples are tested.

Careful storage of degradable supplies following the manufacturer's recommendations and precision in the

Table 5.4 Recommended control organisms for susceptibility testing

Organism	Susceptibility test(s)
<i>Escherichia coli</i> , ATCC 25922	Gram-negative agents
<i>Escherichia coli</i> , ATCC 35218	β -Lactamase inhibitor agents
<i>Staphylococcus aureus</i> , ATCC 25923	Gram-positive agents—Kirby-Bauer test
<i>Staphylococcus aureus</i> , ATCC 29213	Gram-positive agents—minimal inhibitory concentration
<i>Pseudomonas aeruginosa</i> , ATCC 27853	Monitors Ca^{2+} and Mg^{2+} content in media ^a
<i>Enterococcus faecalis</i> , ATCC 29212	Monitors thymidine content in media ^b

^aAs Ca^{2+} and Mg^{2+} concentrations increase, *P. aeruginosa* becomes more resistant to the aminoglycosides.

^bIncreases in thymidine concentration cause false resistance to certain drugs, such as sulfonamides, trimethoprim, and trimethoprim-sulfamethoxazole.

implementation of recommended procedures are mandatory to obtain accurate and reproducible susceptibility results. In any susceptibility system, many variables can affect the accuracy of results, including the following:

- Antimicrobial agent potency
- Agar depth (Bauer-Kirby test)
- Evaporation (microtiter dilution)
- Cation content
- pH
- Thymidine content
- Instrument failure
- Inoculum concentration
- Temperature
- Moisture (Bauer-Kirby test)
- Difficulty in determining end points

Susceptibility testing of control organisms is usually conducted daily until precision can be demonstrated with 20 or 30 consecutive days of susceptibility testing using CLSI guidelines. The control organism results must be evaluated before end points are determined on patient isolates. Minimal inhibitory concentrations (MICs) must be within one log dilution of the expected MIC according to CLSI guidelines. Once the 20- or 30-day evaluation has been accomplished, QC organisms may be tested weekly instead of daily. All results from the 20- or 30-day evaluation should be kept as long as the antimicrobial agent is used or for at least 2 years after discontinuation of use of the agent.

Use of stock cultures

To operate a QC program, all laboratories must maintain stock cultures available from many sources:

- Commercial
- Patient isolates
- Proficiency testing isolates
- ATCC

When QC testing appears to have failed, it is usually the stock culture rather than the test itself that has failed. With

repeated subculturing, organisms can mutate. For best results, a stock culture should be grown in a large volume of broth and then divided among enough small freezer vials to last a year. With this technique, a new vial can be removed from the freezer weekly so organisms do not have to be subcultured continually. Before testing, an organism should be subcultured twice after thawing to return it to a healthy state. Media selection for freezing is at the discretion of individual laboratories, but the media should not contain carbohydrates. If organisms use carbohydrates while being maintained, the acid products that result might kill the organisms over time. The following are popular media choices for stock cultures:

- Schaedler broth with glycerol
- Skim milk
- Chopped meat (anaerobes)
- Tryptic soy agar deeps (at room temperature)
- Cystine tryptic agar without carbohydrates

Another popular method of storage is the use of storage beads. Storage beads are contained in a vial containing 1 mL of thioglycolate broth. After the vial has been inoculated with the organism, the broth is removed, and the vial with the beads is stored at -70°C . The advantage of this system is that a single bead can be removed without the entire vial being thawed. Although this form of storage is more convenient, it is more expensive than those mentioned earlier. Organisms should be frozen at -70°C ; alternative storage methods include freezing in liquid nitrogen and lyophilization.

Quality control manual

All policies and procedures for QC should be written in a QC manual and be available to employees at the workstation. The manual must be reviewed, signed at least annually, and revised as needed by a supervisor. If major changes are made to any of the policies and procedures or if new policies and procedures are introduced, a review and approval by the medical directors are required.

Individualized quality control plan

Individualized quality control plan (IQCP) is the policy name for an alternative CLIA QC program that provides laboratories with the opportunity for equivalent quality testing. Laboratories have the options of following CLIA regulations or developing an IQCP. The three required parts of an IQCP are risk assessment, QC plan (QCP), and quality assessment. An IQCP will not necessarily reduce QC, but it does permit laboratories to develop their own QC protocols that recognize technology included in laboratory test systems. An IQCP allows laboratories to customize QC on the basis of unique environments, test systems, and so on. The laboratory must document that the IQCP is based on evidence collected in-house but can incorporate test system manufacturers' quality certificates.

If a laboratory chooses to implement an IQCP, the laboratory medical director must review and approve the IQCP. Once in place, the IQCP monitors laboratory testing and defines investigation and problem solving, leading to adjustment to the IQCP as needed. In microbiology, for example, visual quality checks of media along with

manufacturers' quality certificates could be considered in the risk assessment. A microbiology laboratory could adapt an IQCP that applies to all media or use separate plans for individual media. In the case of laboratory instrumentation (e.g., a continuous-monitoring blood culture system), the laboratory director might choose to use the manufacturer's instructions for QC if they meet or exceed CLIA regulations. The laboratory must document that the test system results support the number and frequency of the QC specified in the IQCP. To ensure ongoing effectiveness of the IQCP, it must be reviewed annually. CAP published a desired IQCP Annual Evaluation Form that is widely adapted for this purpose.

Evaluating and interpreting diagnostic laboratory tests

Recently, tests in clinical microbiology have shifted toward the detection of antigens, protein profiles, and nucleic acids. Although culture remains the gold standard or reference method for most diagnostic purposes, newer, nonculture detection assays offer simplicity and speed for detection of the causative agent. In addition, in these assays the immune status of the patient does not affect test results, as can occur when detecting antibodies. Nonculture, direct antigen detection tests are designed for use in the physician's office and, as such, are easily performed by non-laboratory personnel. Because the test results are available quickly, the physician can make an informed decision regarding patient treatment.

These tests can, however, be misused or their results misinterpreted. Tests with a high sensitivity and specificity may be promoted as extremely reliable diagnostic tests. In certain clinical situations, such tests may not add meaningful information to the diagnosis. It is important to understand the value and limitations of diagnostic tests for optimal health care.

Analytic analysis of tests

The following terms relate to the actual test and are not used in the critical evaluation of a test in the clinical setting. Analytic sensitivity and specificity should not be confused with clinical sensitivity and specificity.

Analytic (technical) sensitivity and specificity

Sensitivity

The analytic sensitivity of a test refers to its ability to detect a particular analyte or small change in its concentration. **Analytic sensitivity** is usually defined at the 95% confidence level ($\pm$ two standard deviations) and may be referred to as the *detection limit*. In microbiology, the **detection limit** may be correlated to the number of colonies in the culture or to the lowest quantity of antigen or antibody a test can detect. For example, the enzyme immunoassay (EIA) is more sensitive than the precipitation test in detecting antibody. The EIA can detect lower concentrations of antibody.

Specificity

A test's **analytic specificity** refers to its ability not to react with substances other than the analyte of interest. In other words, it detects only one analyte.

Accuracy

The degree of conformity of a measurement to a standard or a true value is its accuracy. It is a measure of analytic capability. For example, with the performance standards for antimicrobial disk susceptibility tests, a mean value of several observations is compared with a predetermined standard value or range. If the mean (or range) of control limits found in standard tables is exceeded, a technical systematic error (variation) exists that might lead to misinterpretation of the test results.

Accuracy is a function of two characteristics: precision and bias. **Precision** is the measure of exactness or the degree of refinement with which a test is performed. It is the dispersion of repeated observations caused by random errors. The precision (reproducibility) of a test is usually monitored by the standard deviation. A larger standard deviation indicates greater dispersion of measurements and less precision. Means of the range (maximum to minimum) within sets of observations can provide a rough measure of precision. **Bias** is the difference between this measured value and the true value of the analyte being measured. It is the mean difference of test results from an accepted reference method caused by systematic errors. Precision and bias are determined by studies wherein several investigators or laboratories perform repeated testing of several specimens of known values. The test results are then analyzed by statistical methods to determine the test's precision and bias within and among laboratories.

Clinical analysis of tests

Clinical (diagnostic) sensitivity

Clinical (diagnostic) sensitivity is the proportion of positive test results obtained when a test is applied to patients known to have the disease; thus it is the frequency of positive test results in patients with the disease (true-positive [TP] results). For example, if 100 patients who have gonorrhea are tested for that disease and the test yields positive results in 95 patients and negative results in the other 5, then the sensitivity of the test is 95%, $(95/100) \times 100$. Five of these 100 patients had false-negative (FN) test results.

The highest clinical sensitivity is desired when a disease is serious and when false-positive (FP) results will not lead to serious clinical or economic problems. Sensitivity is expressed as a percentage:

$$\frac{\text{Number of TP results}}{\text{Number of TP} + \text{FN results}} \times 100$$

The sensitivity of a particular test does not change if the test is performed correctly. The sensitivity is determined by multicenter clinical trials whereby the test results, obtained

by the test being performed according to a specific protocol, are compared with results from a reference method. In microbiology, the reference method is usually culture, which in many cases is not absolute. Thus the reported sensitivity for a certain test may differ among laboratories. Evaluating new diagnostic tests is difficult because of imperfect reference methods.

Clinical (diagnostic) specificity

Clinical (diagnostic) specificity is the proportion of negative results obtained when a test is applied to patients known to be free from the disease. Thus it is the frequency of negative test results in patients without the disease (true-negative [TN] results). For example, if 100 patients without gonorrhea are tested for that disease and the test yields negative results in 90 patients and positive results in the other 10, then the specificity of the test is 90%, $(90/100) \times 100$. Of these 100 patients, 10 had false-positive test results.

The highest clinical specificity is desired when the disease is serious but not treatable, when disease absence has psychological or public health value, or when FP results might cause serious clinical or economic problems. Specificity is expressed as a percentage:

$$\frac{\text{Number of TN results}}{\text{Number of TN} + \text{FP results}} \times 100$$

As with sensitivity, the specificity does not change if the test is performed correctly. The specificity is also determined by clinical trials whereby the test results are confirmed by a more definitive test or procedure.

Operational analysis of tests

Although sensitivity and specificity do not change for a given test, the prevalence of the disease and the positive predictive value (PPV) and negative predictive value (NPV) of a test do change. In clinical medicine, these parameters are extremely important to know when one is evaluating a particular test result.

Incidence of disease

Incidence is the number of new cases of a disease over a period (e.g., months, years) and is a measure of events. The incidence rate is usually calculated by dividing the number of infections acquired during a given period by the population at risk for that same time.

Prevalence of disease

Prevalence is the frequency of a disease at a designated single point in time in the population being tested. For example, the prevalence of strep throat in children attending daycare centers will be higher than the prevalence in children who do not attend such centers. The relationship between prevalence (P) and incidence (I) can be seen in the equation

$$P = I \times D$$

where D is the duration of disease from onset (diagnosis) to termination. For chronic diseases, such as cancer, the prevalence is greater than the incidence; for acute diseases, such as gonorrhea, the prevalence is less than the incidence.

The role prevalence plays in determining the PPV and NPV of a test can be seen in the formulas in the next section. To interpret test results properly, laboratorians and physicians must have an understanding or estimate of the prevalence of the disease in the population being tested. Prevalence can be estimated on the basis of clinical experience and information provided by local and state health departments as well as information periodically provided by the Centers for Disease Control and Prevention.

Predictive values of tests

A diagnostic test has a PPV and an NPV. Three elements are needed to compute the PPV and NPV of a test: the sensitivity and specificity of the test and the prevalence of the disease being tested. The formulas for calculating PPV and NPV are commonly referred to as **Bayes's theorem**, which was published posthumously in 1763.

The **predictive value** of a test is the probability that a positive result (**positive predictive value** [PPV]) accurately indicates the presence of an analyte or specific disease, whereas a negative result (**negative predictive value** [NPV]) accurately indicates the absence of an analyte or specific disease. Predictive values vary significantly with the prevalence of the disease or analyte unless the test is 100% sensitive (for NPV) or specific (for PPV).

Positive predictive value

The PPV is computed as follows:

$$\text{Number of TP results} = (P)(Se)$$

$$PPV = \frac{(P)(Se)}{(P)(Se) + [(1 - P)(1 - Sp)]} \times 100$$

where P is the prevalence of the disease being tested, Se is the sensitivity of the test, and Sp is the specificity of the test. The number of TP plus FP results equals $(P)(Se) + [(1 - P)(1 - Sp)]$.

Negative predictive value

The NPV is computed as follows:

$$\text{Number of TN results} = (1 - P)(Sp)$$

$$NPV = \frac{(1 - P)(Sp)}{(1 - P)(Sp) + (1 - Se)P} \times 100$$

where P is the prevalence of the disease being tested, Se is the sensitivity of the test, and Sp is the specificity of the test. The number of TN plus TP results equals $[(1 - P)(Se)] + [(1 - Se)(P)]$.

Example

To illustrate the concepts of predictive values, consider a diagnostic test that has a sensitivity (Se) of 92% and a

specificity (Sp) of 95%. In a primary care hospital in which the prevalence of the disease being tested for is 1%, the PPV of the test is only 15.7%. This is calculated with the following equation:

$$\text{PPV} = \frac{(0.01)(0.92)}{(0.01)(0.92) + (1 - 0.01)(1 - 0.95)} \times 100 = 15.7\%$$

The primary care provider has only 15.7% certainty that a patient with a positive test result actually has the disease. If, however, the same test is used in a tertiary care hospital in which the prevalence of the disease is 50%, the PPV increases to 95% (calculated with the same equation). The primary care provider in this case has 95% certainty that a patient with a positive test result actually has the disease. Therefore the prevalence of the disease has a great influence on the predictive value of a test.

Clinical applications of positive and negative predictive values

Group A streptococci testing of throat samples

Acute pharyngitis is one of the most common conditions seen by primary care providers. Although most of the infections are caused by viruses, about 15% of cases have a bacterial cause, usually group A β -hemolytic streptococci (*Streptococcus pyogenes*). To test for this organism, 28 million to 36 million throat cultures are performed annually in the United States.

Approved nonculture tests for use in the private office setting are available to evaluate patients with acute pharyngitis for the presence of group A streptococci. Compared with throat culture, the reported sensitivities and specificities of these tests differ considerably. Sensitivities range from 70% to 94%. Specificities have less variation, from 90% to 99%. Although speculative, explanations for such variations may be the difficulty in obtaining an adequate throat sample, especially in children, and the use of imperfect culture methods as standards.

Assuming that a certain test for group A streptococci has reported a sensitivity and specificity of 90% and 98%, respectively, and that the estimated prevalence for streptococcal infection in acute pharyngitis cases is 5%, a typical value for adult populations, the PPV and NPV would be 70.3% and 99.5%, respectively. With a positive test result, approximately a 30% (100%–70.3%) chance exists that the patient does not have a streptococcal infection (FP). If the test result is negative, a greater than 99% chance exists that the patient is not infected. If the prevalence in the population being tested increases to 15%, a typical value for childhood populations, the PPV increases to 88.8%, but the NPV decreases slightly to 98.2%. Because the NPV is less affected by the prevalence

compared with the PPV, the NPV is generally more accurate in predicting the absence of a disease than is the PPV in predicting the presence of a disease. However, if the sensitivity is low, this will adversely affect the results of the NPV.

Direct detection of *Chlamydia trachomatis* in urethral and cervical specimens

Chlamydia is a common sexually transmitted disease (STD) in the United States, with estimates of 3 million to 5 million new cases occurring annually. Many infected individuals are asymptomatic, and proper diagnosis and treatment are essential to prevent the spread of disease and associated complications.

C. trachomatis is an obligate intracellular parasite that requires living cells for cultivation. Several days are necessary to obtain results, and the sensitivity of the culture is only about 85%. As a result, nonculture methods, such as EIA, immunofluorescence (IF), and deoxyribonucleic acid (DNA) probes, are widely used. Each has a considerable variance in reported sensitivities and specificities, most likely because of the nature of the organism and the imperfect culture standard with which the results are compared. In addition, each test requires specialized equipment that must be maintained and calibrated.

Table 5.5 shows the PPVs and NPVs for the IF and DNA probe tests applied directly to cervical samples in a population of patients in an obstetrics and gynecology clinic in whom the prevalence of chlamydial cervicitis was estimated to be 5%. The sensitivities and specificities for the two tests are similar, but the PPV is better for the DNA probe test. Thus patients with positive results of the DNA probe test are more likely to have chlamydial cervicitis than those with positive results of the IF test. On the other hand, both tests identify patients with negative test results as not having chlamydial cervicitis with a high degree of certainty (99.5%).

Table 5.5 also shows the predictive values if the same tests are used in an STD clinic in which the prevalence of chlamydial cervicitis is estimated to be 30%. The PPVs increase significantly, especially for the IF test, and both tests would function well as diagnostic tools. The NPVs drop for both tests, however, to 95.8%. Thus approximately 4% of patients with negative test results could be infected with *C. trachomatis*; this rate may not be acceptable given the nature of the disease.

Efficiency of tests

The efficiency of a test indicates the percentage of patients who are correctly classified as having disease or not having disease. The efficiency is calculated with the following equation:

$$\text{Efficiency} = \frac{(\text{TP} + \text{TN})}{(\text{TP} + \text{FP} + \text{TN} + \text{FN})} \times 100$$

Table 5.5 Comparison of immunofluorescence and DNA probe tests to detect chlamydial cervicitis^a

Test	Sensitivity	Specificity	5% Prevalence		30% Prevalence	
			PPV	NPV	PPV	NPV
IF probe	90.0	98.0	70.3	99.5	95.1	95.8
DNA probe	89.8	99.5	90.4	99.5	98.7	95.8

^aPPV and NPV were computed with equations (see text). All values are given in percentages.

IF, Immunofluorescence; NPV, negative predictive value; PPV, positive predictive value.

where *TP* is the number of patients with TP results, *TN* is the number of patients with TN results, *FP* is the number of patients with FP results, and *FN* is the number of patients with FN results. The Case in Point illustrates the importance of reflex testing, performing an additional test on the basis of the result of another test.

Case check 5.2

The Case in Point illustrates the importance of reflex testing, performing an additional test based on the result of another test. Knowing the sensitivity, specificity, PPV, and NPV of a diagnostic test can lead to improved health care. Because the sensitivity of many rapid streptococcal tests is relatively low (90%) and the proportion of acute pharyngitis cases caused by group A streptococci in children is high (15% to 30%), negative results of rapid tests in children between 5 and 15 years of age should be confirmed by a throat culture. The laboratory in this case enacted the protocol to confirm all negative results in children with a throat culture. It is not necessary to confirm negative results in adults because of a lower prevalence in this population.

Choosing a laboratory method

Once an institution has made the decision to offer a new test, the next step is generally for the laboratory to select the method (Fig. 5.8). The following are some steps that may be followed when deciding on the method:

1. Define the purpose for which the method is used.
Common purposes for tests include:
 - **Screening:** Screening is used for testing asymptomatic populations of patients. Generally, screening tests have high clinical sensitivity and NPV. Positive results with such tests generally require confirmation by a more specific test. The primary purpose of screening is to identify early stages of a disease or health problem to implement treatment.
 - **Surveillance:** Surveillance is a systematic process of identifying, collecting, analyzing, summarizing, and evaluating data about specific diseases or health problems. A large population of individuals are tested; however, the test results are not necessarily returned to the individuals. The data are used by public health officials. Cumulative information is provided to health care providers and the public.
 - **Confirmation:** Confirmation is used after a positive screening result has been obtained to ensure the accuracy of the initial result. Specificity and PPV are generally the considerations for such tests.
 - **Diagnosis:** Diagnosis is used for the evaluation of those suspected of having a given disease state or characteristic.
2. Decide which type of analyte (e.g., organism, antigen, nucleic acid) is to be detected.
3. In conjunction with the end user of the test (e.g., the physician) and information from steps 1 and 2, determine the medical usefulness of the test (e.g., to improve patient care, to shorten hospitalization time).

4. Survey the technical and medical literature for performance claims of various methods. When reviewing the literature, confirm that the method described is actually the test to be evaluated in the laboratory.
5. Other considerations include:
 - Cost
 - Practicality
 - Specimen requirements
 - Quantities of reagents and controls needed for the test
 - Shelf life of reagents and controls before and after opening
 - Availability of supplies, service, and technical support
 - Possible safety hazards
 - Whether the reference range is appropriate for that test and how it will be determined for that institution
 - Whether new equipment needs to be purchased to perform the test
6. Perform an in-house verification. Verification of a test serves to establish that the performance parameters of the test are satisfactory. The result of test verification should indicate one of three possibilities:
 - The test is acceptable for routine use.
 - Further verification studies are required.
 - The test is unsuitable for routine use until its performance parameters can be verified.

Test verification records must be kept for at least 2 years. However, it is good laboratory practice to maintain the records for as long as the test is in use.

Test validation

Verification of a test does not provide ongoing assurance that the test is continually performing as expected. **Test validation** is the ongoing process that provides information that a test is performing correctly. The components of validation are QC, proficiency testing, verification of employee competency, and instrument calibration. The results of the validation indicate one of three possibilities:

1. The test continues to be acceptable.
2. Further investigation is warranted.
3. Immediate corrective action must be undertaken, and the test must be considered unsuitable for routine use until it can be validated.

Lot numbers and expiration dates should be documented for all reagents and materials used in the validation process. Records of validation should be kept for at least 2 years. Validation should be done frequently enough to ensure the continual correct performance of tests. In most cases, following the manufacturer's guidelines and requirements of the regulatory or accrediting agencies will provide this assurance.

New laboratory tests and old ones should always be validated. Understanding and using the concepts of predictive values and the effect of prevalence on those values are important for the proper use of a test and interpretation of the results. The development of rapid nonculture tests for detecting infectious diseases makes the use of these concepts even more important. A test result can no longer be

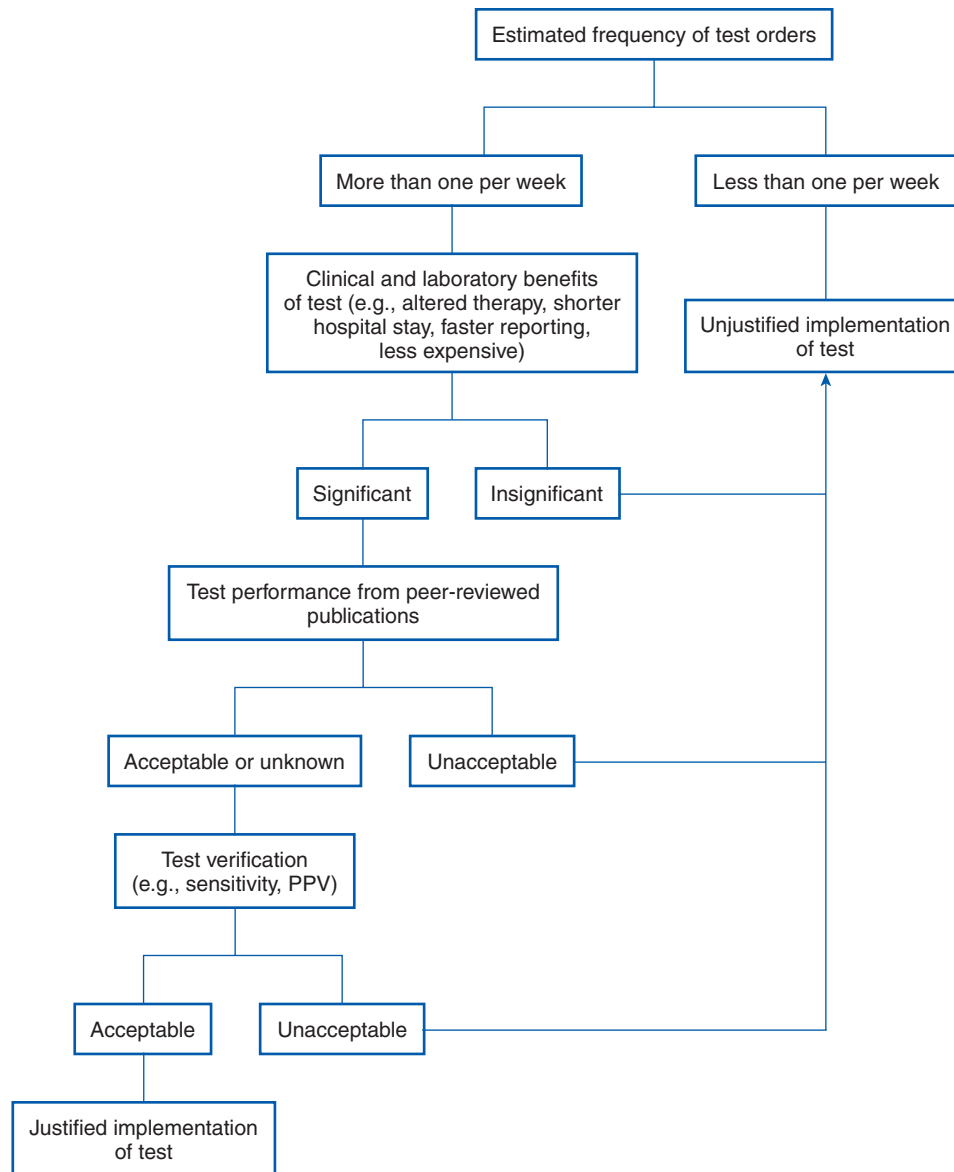


Fig. 5.8 Choosing a laboratory method. PPV, Positive predictive value. (Modified from Clark, R. B., et al. [2009]. *Cumitech 31 A, Verification and validation of procedures in the clinical microbiology laboratory*. In S. E. Sharp (Ed.). Washington, DC: ASM Press.)

considered simply positive or negative but must be interpreted in view of the concepts presented in this chapter. When test results are interpreted properly, better patient care and outcomes are achieved.

POINTS TO REMEMBER

- Quality patient care is directly attributed to the quality of all of the processes involved in that care.
- The laboratory can ensure reliability of laboratory data through the implementation of an active QA program.
- Performance improvements and performance measurement allow organizations to monitor their performance continually and provide opportunities to improve.
- Customers must be considered in quality assurance because all customers will have their own perception of what quality means.
- When outcomes are less than desirable, the entire process must be reviewed and may require cross-functional teams and a trained facilitator to achieve the desired outcome.
- Lean is to minimize waste while at the same time maximizing customer value.
- Six Sigma's goal is to improve quality by reducing variation in any given process and therefore reduce errors in that process.
- Analytic specificity of a test is its ability not to react with substances other than the analyte of interest.
- Clinical or diagnostic specificity is the proportion of negative test results in patients without disease (true-negative results).
- Clinical or diagnostic sensitivity is the proportion of positive test results in patients with disease (true-positive results).
- Incidence is the number of new cases of a disease over a period; prevalence is the frequency of a disease at a designated single point in time.
- Predictive values are influenced by the prevalence of the disease or analyte unless the test is 100% sensitive (for NPV) or specific (for PPV).

LEARNING ASSESSMENT QUESTIONS

1. Which of the following terms refers to checking media and reagents with specific organisms to determine whether expected results are obtained?
 - a. Preventive maintenance
 - b. Quality control (QC)
 - c. Performance improvement (PI)
 - d. Total quality management (TQM)
2. Which of the following characteristics describes the process of performance improvement (PI)?
 - a. It involves only preanalytic activities.
 - b. It is measured by patient outcome.
 - c. It singles out individuals with poor performance.
 - d. It is enhanced by understanding customer perception.
3. The laboratory must perform quality control (QC) on all of the following media except which one?
 - a. Complex media
 - b. Media made by the laboratory
 - c. Media with a history of failure
 - d. All media obtained from a commercial source
4. Which of the following describes the correct way to select organisms for QC?
 - a. They should represent the most fastidious organisms for which the medium was designed.
 - b. They should be organisms that will grow most easily.
 - c. They should be immediately removed from the freezer.
 - d. Streaking should be done only once after their removal from the freezer.
5. Susceptibility tests must be quality controlled daily except when which of the following is the case?
 - a. An automated system is in use.
 - b. Controls have been in an acceptable range for 6 months.
 - c. Precision is demonstrated for 20 or 30 consecutive days.
 - d. A new antimicrobial agent is added.
6. Which of the following mandates annual employee competency testing?
 - a. Clinical and Laboratory Standards Institute (CLSI)
 - b. Clinical Laboratory Improvement Act of 1988 (CLIA-88)
 - c. National Institute of Standards and Technology (NIST)
 - d. American Type Culture Collection (ATCC)
7. A problem-action form should contain which of the following information?
 - a. Date and problem
 - b. Evaluation and corrective action
 - c. Outcome
 - d. All of the above
8. What is the term for performance improvement (PI) monitors created in response to a specific issue?
 - a. Focused monitors
 - b. Process monitors
 - c. Outcome monitors
 - d. Multitask monitors
9. Who is defined as a customer in the laboratory?
 - a. The patient
 - b. The physician and nurse
 - c. The insurance company
 - d. All of the above
10. What is The Joint Commission (TJC) initiative to monitor performance measurement?
 - a. Performance improvement (PI)
 - b. Total quality management (TQM)
 - c. Q-Probes
 - d. Continuous quality improvement (CQI)
11. Which of the following refers to the ability of a test to detect a particular analyte?
 - a. Analytic specificity
 - b. Analytic sensitivity
 - c. Efficiency of tests
 - d. Test validation
12. Using the formula provided in the chapter, determine the positive predictive value (PPV) for a *Chlamydia* test in a population in which the prevalence of the disease is 15%. The sensitivity and specificity of the test are 95% and 98%, respectively.
13. If 100 individuals without syphilis were tested for the disease and 95 tested negative, what is the diagnostic specificity of the test?
14. What parameter would be used to determine the percentage of patients who are appropriately classified as having a disease or not having a disease?
15. Which of the following statements describes the organization's purpose and what it does?
 - a. Vision statement
 - b. Mission statement
 - c. Values
 - d. Customer concept

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Specimen collection and processing

Lauren Roberts

CHAPTER OUTLINE

Basic principles of specimen collection, 124

- Fundamentals, 124
- Collection procedures, 124
- Patient-collected specimens, 124
- Labeling and requisitions, 126
- Safety, 127

Preservation, storage, and transport of specimens, 127

- Specimen storage, 127
- Preservatives, 128
- Anticoagulants, 128
- Holding or transport media, 128
- Shipping infectious substances, 128

Specimen receipt and processing, 130

- Specimen priority, 130
- Unacceptable specimens and specimen rejection, 130
- Macroscopic observation, 131
- Microscopic observation, 131
- Primary inoculation, 131
- Specimen preparation, 132
- Isolation techniques, 132
- Incubation, 132

Culture workup, 134

- Nonroutine specimens, 136

Communication of laboratory findings, 136

Bibliography, 138

OBJECTIVES

After reading and studying this chapter, you should be able to:

1. Identify the role of specimen management in the preanalytic laboratory process.
2. Outline the fundamentals of specimen collection.
3. State the goal of specimen preservation, storage, and transport to the laboratory.

4. Determine when preservatives and anticoagulants are used.
5. Justify the appropriate conditions for storage of specific specimen examples, such as urine and fecal samples.
6. Defend the prioritization guidelines used during processing to prevent degradation of the specimen.
7. Analyze situations in which specimens are unacceptable, and summarize the action to be taken.
8. List the characteristics that can be noted from a macroscopic observation of the specimen.
9. Summarize the purposes of a direct microscopic examination, and identify specimen sources for which this technique is utilized and specimens for which the technique is not beneficial and not performed.
10. Compare the categories of media used in clinical microbiology, and explain how media are selected.
11. Determine the appropriate isolation technique to be used for each specimen source when inoculating solid media.
12. Specify the appropriate temperature and atmospheric conditions for incubation of routine specimens and to recover fastidious bacteria.
13. Summarize the steps involved in the culture workup and interpretation.
14. Devise a plan for processing nonroutine specimens.
15. Assess the significance of the communication of microbiology findings and the role of the laboratory in the postanalytic process.

KEY TERMS

Aerobe	Enrichment broth
Anaerobic transport system	Etiologic agent
Broth media	Expectorated sputum
Capnophile	Homogenization
Cary-Blair transport medium	Induced sputum
Clean-catch midstream urine specimen	Isolation streak
Differential media	JEMBEC system
Direct microscopic examination	Macroscopic observation
Enriched media	Microaerophile
	Nonselective media

Obligate anaerobe
Quantitative isolation
Selective media

Sodium polyanethol sulfonate (SPS)
Suboptimal specimen
Transport media

Case in point

Specimens collected in the pulmonary medicine department were delivered to the microbiology laboratory. In the specimen receipt area, it was noted that one of the bronchoscopy specimens had a loose lid, and a portion of the contents were spilled into the bag. The specimen was submitted in a double bag, and the outer bag appeared dry.

Issues to consider

After reading the technologist's case history, consider:

- The role of the laboratory in assessing the acceptability of specimens received
- The consequences of processing inadequate or improperly collected and transported specimens
- The steps that must be taken in rejection of specimens

A major goal of the microbiology laboratory is to aid in the diagnosis of infectious diseases. Appropriate specimen selection, collection, and transportation are critical if laboratory results are used to provide information that establishes a diagnosis and successful treatment. The microbiology laboratory scientist does not usually perform this preanalytic portion of the laboratory testing process, and yet it directly affects the test results. The data generated by the laboratory are influenced by the quality of the specimen and its condition when received. A poor specimen may result in failure to detect the infectious agent responsible for the patient's condition. It also may result in the administration of inappropriate therapy if treatment is given for a contaminant organism. Thus it is the responsibility of the laboratory scientist to ensure that appropriate specimen management is performed. The laboratory should establish procedures for specimen management, and these procedures must be distributed to all users and clients of microbiology laboratory services. A well-written handbook should be available at every patient care unit and should specify the policies for specimen collection and transport as well as test ordering. Training and education, such as in-service classes taught by the laboratory scientist, should be provided to the individuals collecting the specimens. The laboratory scientist must recognize and reject suboptimal specimens and educate other members of the medical team. Open communication between the laboratory scientist and other members of the medical support team is necessary for quality patient care. This chapter introduces the concepts of specimen collection and processing. The steps for ensuring specimen quality are discussed, along with the procedures to follow when suboptimal specimens are received. This chapter addresses the steps that follow specimen receipt and are involved in completing the processing for microbiology workup.

Basic principles of specimen collection

The laboratory can make accurate and useful determinations only if the correct specimen has been collected properly. The specimens to be analyzed are likely to contain living

organisms; the goal of the specimen collector is to maintain the viability of these organisms with minimal contamination.

Fundamentals

The following basic principles of specimen collection are fundamental to ensuring appropriate specimen management:

- If possible, collect the specimen in the acute phase of the infection and before antimicrobial agents are administered.
- Select the correct anatomic site for collection of the specimen.
- Collect the specimen using the proper technique and supplies with minimal contamination from normal biota (flora).
- Collect the appropriate quantity of specimen.
- Package the specimen in a container or transport medium designed to maintain the viability of the organisms and avoid hazards that result from leakage.
- Label the specimen accurately with the specific anatomic site and the patient information—patient's name and a unique identification number, as well as the date and time of collection.
- Transport the specimen to the laboratory promptly, or make provisions to store the specimen in an environment that will not degrade the suspected organism(s).
- Notify the laboratory in advance if unusual pathogens or agents of bioterrorism are suspected.

Collection procedures

Specimens for microbiology cultures should be collected in sterile containers except for stool specimens, which can be collected in clean, leakproof containers. Generally, swabs are not recommended for collection because they do not provide sufficient quantity, are easily contaminated, and can become dried out, leading to a loss of organisms. Swabs are appropriate for specimens from the upper respiratory tract, external ear, eye, and genital tract. The tips of swabs may contain Dacron, rayon, or calcium alginate. Cotton-tipped swabs tend to have excessive fatty acids that may be toxic to certain bacteria and are not recommended. Dacron or rayon polyester swabs have a wide range of uses. Swab collection systems are available that provide **transport media** and protect the specimen from drying.

Lesions, wounds, and abscesses present many problems to the microbiology laboratory. A wound is not an appropriate specimen source when the exact anatomic site is not provided. Before the specimen is collected, the area should be cleansed to eliminate as much of the commensal biota as possible. The specimen should be collected by needle aspiration whenever possible, rather than by swab from the advancing margin of the lesion. Aspirated material should be placed into a sterile tube or vial with transport media and not "squirted" onto a swab. [Table 6.1](#) lists specimen collection procedures.

Patient-collected specimens

In certain situations, patients are asked to collect the specimen themselves. Medical personnel should provide patients with thorough instructions on how to collect the sample and should not assume that the patient knows how to collect a particular type of specimen. Printed instructions in multiple

Table 6.1 Specimen collection guidelines

Specimen	Patient preparation	Container/Minimum quantity
Blood culture	Disinfect skin with alcohol and iodine or chlorhexidine before venipuncture	Blood culture media set (aerobic and anaerobic bottles) or Vacutainer tube with SPS, adults 20 mL per set, children 5–10 mL per set
Body fluids (abdominal, amniotic, ascites, bile, joint, pericardial, pleural)	Disinfect skin before needle aspiration	Sterile, screw-cap tube or anaerobic transport system, ≥ 1 mL
Catheter tips, IV (Foley catheters not cultured)	Disinfect skin before removal of catheter	Sterile, screw-cap container
Cerebrospinal fluid	Disinfect skin before aspiration	Sterile, screw-cap tube, bacteria ≥ 1 mL, fungi ≥ 2 mL, AFB ≥ 2 mL, virus ≥ 1 mL
Ear		
Inner	Clean ear canal with mild soap, aspirate fluid with needle if eardrum intact; use swab if eardrum is ruptured	Sterile, screw-cap tube or anaerobic transport system
Outer	Remove debris or crust from ear canal with saline-moistened swab; rotate swab in outer canal	Swab transport system
Eye		
Conjunctiva	Sample both eyes; use separate swabs moistened with sterile saline	Swab transport system
Corneal scrapings	Instill local anesthetic, scrape with sterile spatula and inoculate directly to agar	Agar available at bedside
Feces	Collect directly into container, avoid contamination with urine	Clean, leakproof container or enteric transport system
Fungal scrapings	Wipe nails or skin with alcohol	Clean, screw-cap container
Hair/nails/skin	Hair: 10–12 hairs with shaft intact Nails: Clip affected area Skin: Scrape skin at outer edge of lesion	
Genitalia		
Cervix/vagina	Remove mucus before collection; do not use lubricant on speculum; swab endocervical canal or vaginal mucosa	Swab transport system or JEMBEC transport system, Assay specific system if NAAT performed
Urethra	Flexible swab inserted 2–4 cm into urethra for 2–3 seconds or collect discharge	Swab transport system or JEMBEC transport system, Assay specific system if NAAT performed
Lesion/wound/abscess	Wipe area with sterile saline or alcohol	
Superficial	Swab along outer edge	Swab transport system
Deep	Aspirate with needle and syringe	Anaerobic transport system
Respiratory tract: lower		
Sputum	Rinse mouth or gargle with water, instruct to cough deeply into container	Sterile, screw-cap container
Bronchial specimens	Materials collected during bronchoscopy	Sterile, screw-cap container
Respiratory tract: upper		
Nasal	Insert premoistened swab with sterile saline 1 inch into nares	Swab transport system
Nasopharynx	Insert flexible swab through nose into posterior nasopharynx, rotate for 5 seconds	Swab transport system or direct inoculation to media
Throat	Swab posterior pharynx, tonsils, and inflamed areas	Swab transport system
Tissue	Disinfect skin; do not allow tissue to dry out; if necessary, moisten with sterile saline	Anaerobic transport system or sterile screw-cap container
Urine		
Clean-catch midstream	Cleanse external genitalia; begin voiding; after several mL have passed, collect midstream without stopping flow of urine	Sterile, screw-cap container or urine transport kit, 2–3 mL

Continued

Table 6.1 Specimen collection guidelines—cont'd

Specimen	Patient preparation	Container/Minimum quantity
Catheter	Cleanse urethral area, insert catheter, and allow first 15 mL to pass; collect remainder	Sterile, screw-cap container or urine transport kit
Indwelling catheter	Disinfect catheter collection port, aspirate 5–10 mL with needle and syringe	Sterile, screw-cap container or urine transport kit
Suprapubic aspirate	Disinfect skin, aspirate with needle and syringe through abdominal wall into full bladder	Sterile, screw-cap container or anaerobic transport system

AFB, Acid-fast bacilli; IV, intravenous; JEMBEC, agar plates for transporting cultures of gonococci; NAAT, nucleic acid amplification test; SPS, sodium polyanethol sulfonate.

languages attached to a collection device, combined with verbal instructions, is the most effective method. It may be necessary to read the instructions to the patient. The instructions should be written in simple language and pictures included to help the patient understand the procedure as it is verbally explained. The specimens commonly obtained by the patient are urine, sputum, and stool.

Urine

Instructions for urine collection must include an explanation of what a **clean-catch midstream urine specimen** means. A first morning specimen is preferred because it provides a more concentrated sample. The patient collects this specimen following cleansing of the external genitalia to reduce the presence of indigenous biota. Patients are asked to void without collecting the first portion of the urine flow and instead to collect the middle portion. The first portion of the urine flow washes contaminants from the urethra, and the midstream portion is more representative of the urine in the bladder. Personnel who collect catheterized specimens should also use this technique to eliminate organisms carried up the urethra during catheterization.

Sputum

Sputum specimens are often collected for the diagnosis of bacterial pneumonia. Lower respiratory tract specimens are among the most difficult specimens to collect adequately because they are contaminated with oropharyngeal biota. For this reason, they are one of the least clinically relevant specimens received for culture. Other specimens, such as blood or a bronchoalveolar lavage (BAL), may be more accurate in detecting the **etiologic agent** (i.e., the microorganism causing the disease).

Collection of a quality sputum sample requires thorough patient education and medical personnel oversight of the process. The first early morning specimen is preferred. The patient must understand the difference among sputum, saliva, and nasal secretions. Patients should rinse their mouth with water and expectorate with the aid of a deep cough directly into a sterile container (**expectorated sputum**). Patients with dentures should remove the dentures first. A single specimen should be adequate for detection of a bacterial lower respiratory tract infection. If fungal or mycobacterial infections are suspected, three separate early morning specimens are appropriate.

Respiratory therapy technicians may assist patients who are unable to expectorate a respiratory specimen. These

specimens may be collected through aerosol induction in which the patient breathes aerosolized droplets of a solution that stimulates cough reflex (**induced sputum**). When sputum specimens are submitted to microbiology, the laboratory should be informed of whether the specimen was expectorated or induced.

Stool

The specimen of choice for the detection of gastrointestinal pathogens is stool. A rectal swab can be submitted for bacterial culture as long as fecal material is visible on the swab. A negative culture result for bacterial pathogens may require the submission of a second specimen. If a parasitic infection is suspected, three specimens collected within 10 days should be sufficient for microscopic detection of ova and parasites. Some laboratories offer an initial parasite screening for *Giardia lamblia* and *Cryptosporidium* spp. Newer methods detect parasite antigens, and one sample is usually sufficient. If the screen is negative, the physician can decide whether to perform complete parasite studies.

Patients should be instructed to defecate directly into the collection device. Specimens should never be taken from the toilet and should not be contaminated with urine. Commercial systems are available with preservatives for bacteria and parasites. The appropriate ratio of stool to preservative is 1:3, and the patient must understand that if this ratio is not met, the test will be invalid. Commercial transport containers are labeled with fill lines to aid in achieving the appropriate ratio. In addition, the patient needs to be told that the specimen must be thoroughly mixed with the preservative. Specimens for parasite microscopic studies should be collected before any barium studies are done. If this is not feasible, the patient must delay specimen collection until the barium has cleared (4 to 5 days). Barium appears as a white chalky substance in the specimen and masks the appearance of parasites under the microscope.

Culture-independent methods are becoming more available in laboratories. These molecular assays are highly sensitive and do not require multiple specimens. The collection container and transport will be specified by the assay manufacturer. Patients must be provided the instructions and collection devices.

Labeling and requisitions

It is important that correct patient identification be put on the specimen container and the requisition. The specimen label must contain sufficient information for the specimen and

requisition to be matched when received in the laboratory. The laboratory loses valuable time when specimens are unlabeled or mislabeled. Resolution requires making phone calls and filling out additional paperwork, and it ultimately delays processing the specimen or requires that a new specimen be collected. This may ultimately delay the diagnosis.

Proper identification of each specimen includes a label firmly attached to the container with the following information:

- Full patient name
- Date of birth
- Identification number
- Room number or location
- Physician
- Culture site
- Date and time of collection
- Name or initials of collector

To perform quality laboratory analysis, the laboratory needs specific information regarding the patient and the specimen. All that the laboratory knows about the patient is learned from the requisition form. The less information that is provided, the more difficult it is for the laboratory to provide good patient care. Incomplete information on the requisition form is often a weak link in the specimen management process. The requisition form should provide the following information:

- Patient's name
- Patient's age (or date of birth) and gender
- Patient's room number or location
- Physician's name, address, and phone number
- Specific anatomic site
- Date and time of specimen collection
- Clinical diagnosis or relevant patient history
- Antimicrobial agents (if patient is receiving any)
- Name of individual transcribing orders

Complete and thorough requisitions can often lead the laboratory scientist to suspect certain pathogens based on the diagnosis or patient history. This knowledge can allow use of specific media or making certain adjustments to the incubation to maximize recovery of the pathogen.

Computer-based ordering is performed at many institutions. Ideally, the laboratory scientist should design the test-ordering process; this will enable the laboratory to collect the necessary information. These systems should be designed to provide key fields that must be completed to submit the request transaction.

The laboratory scientist should recognize that the individual ordering the test does not have complete knowledge of what are and are not appropriate tests for each specimen. If the test requested is not recommended, it is the responsibility of the laboratory to communicate with the physician to determine exactly what must be done.

Safety

It is imperative that specimens collected for microbiology not pose a safety hazard to the individuals who handle them. Leaking containers and specimens with needles attached

present the greatest hazards. All specimens must be transported in leakproof secondary containers. Because the specimen should be kept separate from any paperwork, plastic bags with permanent seals and separate pouches on the outside for requisitions are recommended.

Transporting personnel should refuse to transport specimens without the protection of a secondary container. Refusing to accept syringes with needles attached is also appropriate. A needle must be replaced with a tight-fitting rubber stopper or a stopcock to put resistance on the plunger. The aspirated material could also be transferred to a sterile container with a tight lid or to an **anaerobic transport system**.

Laboratory personnel must also adhere to strict safety guidelines as they begin to work with the patient's specimen. All individuals handling patient specimens must wear protective clothing, and specimens should be opened only in a biosafety cabinet.

Case check 6.1

The laboratory can make accurate and useful determinations only if a specimen has been collected and transported properly. The goal of the specimen collector must be to maintain the viability of the living organisms and avoid hazards that result from leakage. The Case in Point clearly shows the laboratory's role in assessing the acceptability of the specimens it receives.

Preservation, storage, and transport of specimens

Specimen transport is another essential component of the preanalytic process of microbiology testing. Whether the specimen comes from within the hospital or clinic or from an outpatient facility or physician's office across town, the goal is the same. The primary goal in the transportation of specimens to the laboratory is to maintain the specimen as near to its original state as possible with minimal deterioration and to prevent risk to the specimen handler. If specimen deterioration results in death of the causative agent present or overgrowth of contaminants, the specimen is no longer representative of the disease process.

Specimens should be transported to the laboratory ideally within 30 minutes of collection, preferably within 2 hours. Adverse environmental changes in oxygen, pH, and temperature can prevent the recovery of certain microorganisms and allow overgrowth of others. If transport to the laboratory is delayed or if the specimen will not be processed as it is received in the laboratory, the specimen can be maintained by storage under certain conditions or with the use of preservatives, anticoagulants, transport or holding medium, or culture medium.

Specimen storage

Some specimens that will not be transported or processed immediately can be maintained by being stored under certain conditions. The individual responsible for storing the specimen must be informed as to the best storage

Table 6.2 Specimen storage guidelines

Refrigerate	Room temperature
Catheter tips (IV)	Abscess, lesion, wound
CSF for viruses	Body fluids
Ear: outer	CSF for bacteria
Feces (unpreserved)	Ear: inner
Feces for <i>Clostridioides difficile</i> toxin (up to 3 days; >3 days store at -70°C)	Feces (preserved)
Sputum	Genital
Urine (unpreserved)	Nasal, nasopharynx, throat
	Tissue
	Urine (preserved)

CSF, Cerebrospinal fluid; IV, intravenous.

environment for each specimen type. Some specimens, such as urine, stool, sputum, bronchial secretions, swabs (not for anaerobes), foreign devices such as catheters, and viral specimens, can be maintained at refrigerator temperature (4°C) for 24 hours. Pathogens that are cold sensitive may be found in other specimens, and those specimens should be kept at room temperature if culture is to be performed. This includes samples that might contain anaerobic bacteria as well as most other sterile body fluids, genital specimens, and ear and eye swabs. Fecal specimens submitted in preservatives can be maintained at room temperature. If cerebrospinal fluid is not processed immediately, it can be stored in a 35°C incubator for 6 hours. Table 6.2 lists specimen storage guidelines.

Preservatives

Two specimen types in which preservatives can be used are urine and stool. Boric acid is used in commercial products to maintain accurate urine colony counts. The systems are designed to maintain the bacterial population in the urine at room temperature for 24 hours and are useful for collection of urine specimens at distant locations. Stool specimens for bacterial culture that are not transported immediately to the laboratory can be refrigerated; if the delay is longer than 2 hours, the specimen can be added to **Cary-Blair transport medium**. Stools for *Clostridioides difficile* toxin assay should be collected without a preservative and can be refrigerated. If the delay is expected to be longer than 48 hours, the specimen should be frozen at -70°C . Preservatives for ova and parasite (O & P) examinations maintain the morphology of trophozoites and cysts. Laboratories often use a two-vial system in which one vial contains formalin for concentration and the other vial contains a fixative for preparing stained slides, such as modified polyvinyl alcohol with zinc.

Anticoagulants

Anticoagulants are used to prevent clotting of specimens, including blood, bone marrow, and synovial fluid. Organisms trapped in clotted material are difficult to isolate. The type of anticoagulant used and the concentration are important because some anticoagulants have antimicrobial properties. **Sodium polyanethol sulfonate (SPS)** is the most common anticoagulant used for microbiology specimens. The concentration of SPS must not exceed 0.025% (weight/volume) because some *Neisseria* spp. and certain anaerobes are

inhibited by higher concentrations. The ratio of specimen to SPS is important; different sizes of tubes must be available to accommodate adult and pediatric blood specimens and bone marrow or synovial fluid. Heparin is another acceptable anticoagulant and is often used for viral cultures and for isolation of *Mycobacterium* spp. from blood. Citrate and ethylenediamine tetraacetic acid (EDTA) should not be used for microbiology specimens.

Holding or transport media

Another way to maintain the integrity of the specimen from the time of specimen collection until laboratory processing of the sample is with the use of holding or transport media. These media usually contain substances that do not promote multiplication of microorganisms but ensure their preservation and are available in swab collection systems. Stuart or Amie transport medium is commonly used. Some transport systems contain charcoal to neutralize substances that can be detrimental to the survival of *Neisseria gonorrhoeae* and *Bordetella pertussis*. Flocked swabs (FLOQSwabs; Copan Diagnostics, Murrieta, CA) are also available that utilize materials to improve absorption and release of materials. These have been shown to be superior to wrapped swabs for pathogen recovery.

In certain situations, direct inoculation to culture media at the time of specimen collection (bedside inoculation) is optimal for isolation of the pathogen. Blood is usually placed into a broth culture medium immediately after collection. Synovial and peritoneal fluids also can be inoculated into blood culture broth bottles at the bedside. Besides the blood culture bottles, an additional specimen should be sent to the laboratory in a container for Gram stain. Specimens for *N. gonorrhoeae* can be placed directly onto a commercial transport system such as the **JEMBEC system** (John E. Martin Biologic Environmental Chamber; Thermo Fisher Scientific, Waltham, MA). This system contains selective agar and a carbon dioxide (CO_2)-generating tablet. Nasopharyngeal swabs for the isolation of *B. pertussis* also can be inoculated directly onto selective agar as they are collected. Specimens collected from the eye, especially cornea scrapings, also are inoculated directly to appropriate media as they are collected.

Specimens collected at the bedside are more susceptible to contamination. Any time culture medium is taken outside the laboratory, there is a possibility that it can become contaminated. The individual collecting the specimen must be informed regarding the appropriate way to manipulate the medium. In some situations, the laboratory scientist is asked to assist in the collection and can monitor the appropriate application of the specimen to the culture media. In other situations, media are maintained at the outpatient facility, and the culture is sent to the laboratory already inoculated with the specimen. It is the responsibility of the microbiology laboratory to ensure that the culture medium is of good quality when it is received to isolate the pathogens involved in the infection.

Shipping infectious substances

The shipment of clinical specimens and cultures of microorganisms is governed by a complex set of national and international guidelines issued by the U.S. Department of Transportation (DOT) and the U.S. Postal Service. International air shipment is regulated by the International

Civil Aviation Organization. The U.S. Congress requires the Secretary of Transportation to prescribe regulations for the safe transportation of hazardous material in commerce to ensure public safety and minimize risks in transportation. The regulations specify the way potentially infectious substances must be packaged to prevent leaks or spills and withstand pressure changes as well as how packages must be labeled to caution handlers and other parties about their hazardous content. The goal is to safeguard employees in the transportation industry and the general public. The duty of the laboratory is to use the appropriate packaging for each material being shipped and to label the package properly. Laboratories can purchase packaging materials specifically designed for transporting laboratory specimens.

Infectious substances are considered a hazardous material and must meet the requirements of the DOT Hazardous Material Regulations as published in the *Federal Register* Title 49 Code of Federal Regulations Parts 171 to 180 before being transported by rail, water, air, or highway. The DOT defines an *infectious substance* as a material known or suspected to contain a pathogen (bacteria, viruses, rickettsiae, parasites, fungi, or prions) that causes disease in humans or animals. Infectious substances are classified using a two-tier system: category A and category B. Category A substances are capable of causing permanent disability or life-threatening or fatal disease in otherwise healthy humans or animals upon exposure. Category B substances are not in a form generally capable of causing permanent disability or life-threatening or fatal disease in otherwise healthy humans or animals on exposure. Exposure occurs when the infectious substance is released

outside the protective packaging and results in physical contact with humans or animals.

Patient specimens or culture isolates must be triple-packaged before being shipped. The material is placed into a primary receptacle that must be watertight. Absorbent material is placed around the primary receptacle, and it is then placed into a secondary container that is also watertight. The secondary package is sealed and placed into a sturdy outer container constructed of fiberboard. Specific instructions must be followed for labeling the container as "Hazardous Material." Specimens that are shipped by air require specific labeling and shipping documents. Fig. 6.1 demonstrates the packaging of infectious materials for shipping.

Every employee who packages specimens and infectious materials for shipment must be appropriately trained. Training must include the DOT and International Air Transportation Association regulations; retraining should occur every 2 years if shipping by air or every 3 years if shipping by ground only, unless there are significant changes in the regulations.

Case check 6.2

The primary goal of specimen transport is to maintain the specimen as near to its original state as possible with minimal deterioration while protecting those handling the specimen. The Case in Point demonstrates the need for tightly sealed leakproof containers. Specimens should not be externally contaminated. Specimens that are grossly contaminated or compromised may be rejected.

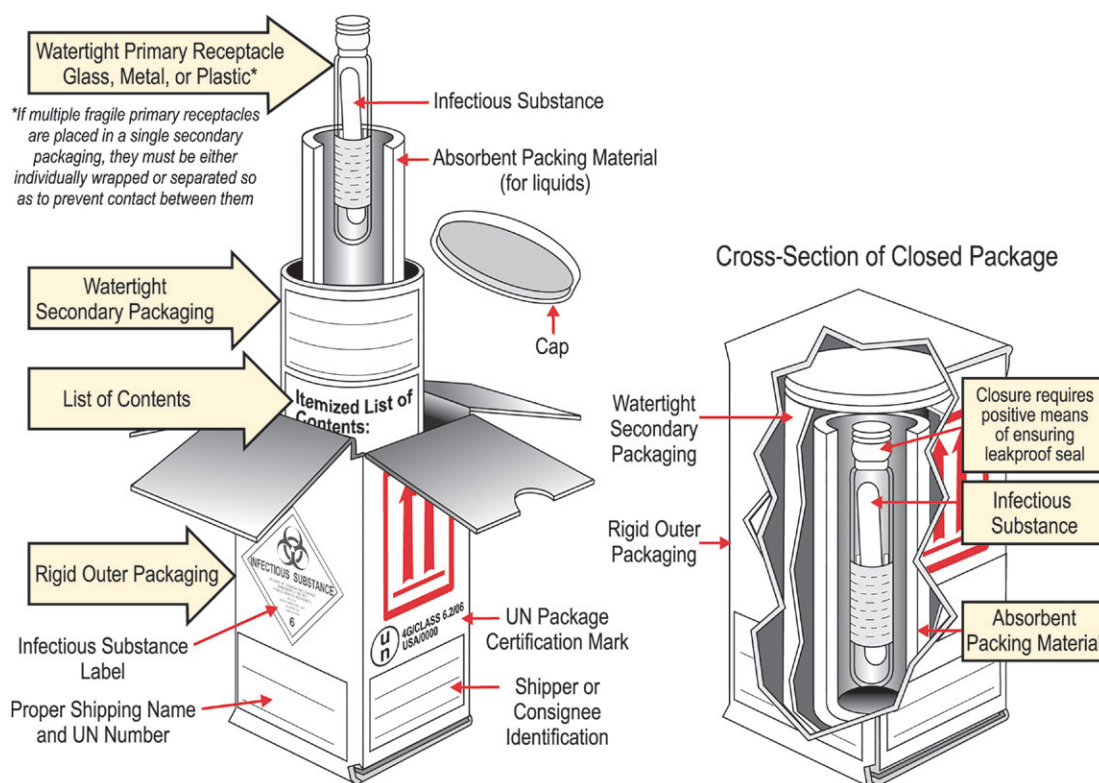


Fig. 6.1 Packaging infectious substances for shipping: Category A Shipping. (From U.S. Department of Transportation, Pipeline and Hazardous Materials Safety Administration. (2019, December 4). *Transporting infectious goods safely*. CDC IPP Webcast. Available at: https://www.cdc.gov/cpr/ipp/webcast-2019/docs/DOT-Transporting-Infectious-Goods-Safely_508.pdf.)

Specimen receipt and processing

Specimen priority

Specimens require prompt processing after arrival in the laboratory. Processing every specimen as soon as it is received is often impossible. Laboratory staffing and specimen load may have a significant effect on timely processing. Appropriate specimen management should include guidelines for prioritizing the handling of specimens. A four-level scheme of prioritization may be used based on the critical nature of the specimen or potential for specimen degradation. Table 6.3 lists clinical samples and the ways each can be prioritized in a four-level system.

Level 1 specimens are classified as critical because they represent a potentially life-threatening illness and are from an invasive source. They require immediate processing. Level 2 specimens are unprotected and may quickly degrade or have overgrowth of contaminating biota. The laboratory scientist must quickly provide an optimal growth environment for the fastidious organisms that may be found in these specimens. Level 3 specimens require quantitation. Delay in processing level 3 specimens may adversely affect the accuracy of quantitation.

If processing of level 2 and level 3 specimens is postponed, appropriate storage or preservation must be initiated. For example, urine and sputum specimens can be refrigerated. A refrigerator at the site of specimen processing is convenient for the laboratory scientist and makes it more likely that urine specimens will be refrigerated during peak workload times. Level 4 specimens are specimens that arrive in the laboratory in holding or transport

media. Processing of level 4 specimens may be delayed to process specimens of a more critical nature first.

In general, batch processing is not used for most specimens in microbiology; however, in a few situations, it is appropriate. Specimens for acid-fast bacilli that need to be digested and decontaminated can be refrigerated and processed once per day. Stool specimens for O & P examinations that are in preservative also can be processed in batch format. Specimens for viral culture that are collected in viral transport media also can be batched.

Unacceptable specimens and specimen rejection

The analytic phase of the laboratory testing process begins as the specimen is received in the laboratory. On receipt in the laboratory, the specimen must be examined to ensure that it has been properly selected, collected, and transported. Performing tests on specimens that are of poor quality would yield misleading information that might result in misdiagnosis and inappropriate therapy. The microbiology laboratory must establish and publish the criteria for specimen rejection. The following situations are examples of **suboptimal specimens** that must be rejected:

- The information on the requisition does not match the information on the specimen label. If the patient name or source does not match, the specimen should be collected again.
- There is no patient identification on the specimen container.
- The specimen is not submitted in the appropriate transport container, or the container is leaking or grossly contaminated externally.
- The quantity of the specimen is inadequate to perform all tests requested.
- The specimen transport time is more than 2 hours, and the specimen has not been preserved.
- The specimen is received in a fixative such as formalin; stools for O & P examinations are an exception.
- An anaerobic culture is requested on a specimen in which anaerobes are indigenous.
- Microbiology processing of a particular specimen results in questionable data (e.g., Foley catheter tip).
- The specimen is dried up.
- More than one specimen from the same source was submitted from the same patient on the same day; blood cultures are an exception.
- One swab was submitted with multiple requests for various organisms.
- Gram stain of expectorated sputum reveals fewer than 25 white blood cells (WBCs) and more than 10 epithelial cells per low-power field and mixed bacterial biota.

All rejected specimens require a phone call to the person in charge of collecting the specimen. The laboratory should never discard an unacceptable specimen before contacting a member of the health care team. The laboratory must document the situation, indicating the reason for the rejection of the specimen. If the physician insists on processing an inadequate specimen, the laboratory report must include a comment explaining the potentially compromised results.

Table 6.3 Levels of specimen prioritization

Level	Description	Specimens
1	Critical/invasive	Amniotic fluid Blood Brain Cerebrospinal fluid Heart valves Pericardial fluid
2	Unpreserved	Body fluids (not listed for level 1) Bone Drainage from wounds Feces Sputum Tissue
3	Quantitation required	Catheter tip Urine Tissue for quantitation
4	Preserved	Feces in preservative Urine in preservative Swabs in holding medium (aerobic and anaerobic)

It may be necessary to process a suboptimal specimen in certain situations. Specimens that are impossible to recollect or that would require the patient to undergo another invasive procedure (bone marrow, spinal fluid, or surgery) may need to be processed regardless of the situation. The final report must include a notation indicating that the specimen was compromised.

Macroscopic observation

Processing patient specimens begins with a **macroscopic observation**. The gross appearance of the specimen may provide useful information to both the microbiologist and the physician. The physical characteristics of the specimen should be documented so that if different laboratory scientists work on the sample, they all will know the results of the gross examination.

Notations from the macroscopic observation should include the following:

- Swab or aspirate
- Stool consistency (formed, loose, or liquid)
- Blood or mucus present
- Volume of specimen
- Fluid—clear or cloudy

The gross examination also allows the processor to determine the adequacy of the specimen and the need for special processing. Areas of blood and mucus are selected for culture and direct microscopic examination. Anaerobic cultures may be indicated if gas, foul smell, or sulfur granules are present. The diagnosis is evident if adult helminths or tapeworm proglottids are present in the specimen.

Microscopic observation

A **direct microscopic examination** is a useful tool that provides rapid information. In critical situations, such as meningitis, the direct microscopic examination can be used to guide therapy choices when therapy must be initiated before culture results are available. Microscopic observation serves several purposes: (1) It can be used to determine the quality of the specimen. Sputum specimens that represent saliva rather than lower respiratory tract secretions can be determined by the quantitation of WBCs or epithelial cells. Similar assessment and determination can be made in samples collected from a wound site. Absence of WBCs may indicate that the sample may not have been taken from the actual site of infection. (2) It can give the laboratory scientist and the physician an indication of the infectious process involved. Gram stain of a sputum specimen revealing WBCs and gram-positive diplococci is indicative of *Streptococcus pneumoniae*. The routine culture workup can be guided by the results of the smear. The laboratory scientist can correlate the bacterial isolates with the types detected in the smear; this may alert the laboratory scientist to the presence of additional organisms not yet growing, such as anaerobes. It can dictate the need for nonroutine or additional testing. The presence of fungal elements in a specimen for bacterial culture would alert the laboratory scientist to notify the physician to request a fungal culture. Specimens may be received in many forms. Preparation of the direct smear depends on the type of material received. Techniques differ according to whether the specimen is a tissue, swab, or

fluid. See [Chapter 7](#) for a detailed explanation of the preparation and staining of smears.

In certain specimen types, the direct microscopic examination does not provide useful information and is not appropriate. Throat and nasopharyngeal specimens are examples. Gram stains for *N. gonorrhoeae* on specimens from the cervix and anal crypts are not recommended because these sites can contain other bacteria that can have the same morphology, although Gram stain direct smears are recommended to diagnose bacterial vaginosis. Gram stains on stool specimens are not usually routine, although they can be useful to determine whether the patient has an inflammatory diarrhea based on the presence of WBCs.

Although direct Gram stains on a urine specimen provide useful information, many laboratories do not perform them routinely because they are time-consuming, and preliminary culture results are available within 24 hours. Physicians can always request that a direct Gram stain be performed.

Primary inoculation

Types of culture media

Culture media may be divided into categories defined by the ability to support bacterial growth. **Nonselective media** support the growth of most nonfastidious microbes. Sheep blood agar is the standard nonselective medium used in the United States. **Selective media** support the growth of one type or group of microbes but not another. A selective medium may contain inhibitory substances such as antimicrobials, dyes, or alcohol. MacConkey agar is selective for enteric gram-negative bacilli, and Columbia agar with colistin and nalidixic acid (CNA) is selective for gram-positive organisms. **Differential media** allow grouping of microbes based on different characteristics demonstrated on the medium (see Chapter 8). Media may be differential and nonselective (e.g., sheep blood agar is nonselective but differentiates organisms on the basis of hemolysis). Media can be differential and selective (e.g., MacConkey agar inhibits gram-positive organisms and differentiates gram-negative bacilli on the basis of lactose fermentation).

Enriched media contain growth enhancers that are added to nonselective agar to allow fastidious organisms to flourish. Chocolate agar is an enriched medium. **Enrichment broth** is a liquid medium designed to encourage the growth of small numbers of a particular organism while suppressing other biota present. Enrichment broths are incubated for a certain period and then must be subcultured to isolate the particular organism. Lim broth (Todd Hewitt broth with CNA) is used to enhance the growth of group B streptococci. **Broth media** can be used as a supplement to agar plates to detect small numbers of most aerobes, anaerobes, and microaerophiles. Thioglycollate broth is an example of a supplemental broth media.

Culture media selection

The selection of media to inoculate is based on the type of specimen submitted for culture and the organisms likely to be involved in the infectious process. Specimens in which fastidious pathogens are more likely involved require media with appropriate nutrients to aid in their recovery. Specimens

that are collected from a site containing normal biota require types of media to diminish the normal biota, while allowing the pathogens to be detected.

Selection of primary culture media is standardized for routine bacterial cultures. However, individual laboratories may prefer one medium to another on the basis of past experience, patient population, or other circumstances. A table of media to be inoculated for each specimen should be available in the specimen processing area. See [Table 6.4](#) for primary culture media selection for specific body sites; equivalent media may be substituted. In some laboratories, the computer generates labels for media as the specimen is accessioned. Some specimens require a single plate, whereas others require a battery of several plates. If several plates will be inoculated, the labeled media should be arranged in order, beginning with the most enriched medium and progressing to the most selective. The specimen can be applied to each culture medium, and inhibitory substances will not be carried over from one agar to another.

The routine primary plating media include the following items:

- Nonselective agar plate.
- Enriched medium for fastidious organisms for normally sterile body fluids or a site in which fastidious organisms are expected.
- Selective and differential medium for enteric gram-negative bacilli for most routine bacterial cultures.
- Selective medium for gram-positive organisms for specimens in which mixed gram-positive and gram-negative bacteria are found.
- Additional selective media or enrichment broths for specific pathogens as needed.
- Broth medium may be used as a supplement with specimens from sterile body fluids (e.g., tissues, lesions, wounds, and abscesses).

Specimen preparation

Most specimens arrive in the laboratory in one of three forms: swab, tissue, or fluid. Specimens such as sterile body fluids, pus, urine, and sputum are inoculated directly onto selected media. Large volumes of sterile body fluids (peritoneal, pleural, continuous ambulatory peritoneal dialysis) are concentrated to increase the recovery of bacteria. Centrifugation and filtration are methods for concentration. If the volume of fluid is greater than 1 mL, the specimen can be centrifuged for 20 minutes at 3000g. The sediment is then used to inoculate media and to prepare smears. If the specimen consistency is thin enough to avoid filter clogging, filtration with a Nalgene filter unit can be performed. After filtration, the filter is removed and placed on the surface of an agar plate.

Specimens received on swabs can be inoculated directly to culture media. The specimen should be submitted on two swabs: one is used for the culture media, and the other is used to make the direct smear. Some laboratories place the swab into 0.5 to 1.0 mL of broth or saline and then vortex the specimen to loosen material from the swab and produce an even suspension of organisms. A sterile pipette is used to dispense the inoculum onto plates and broth. Tissues can be prepared for culture by **homogenization**, in which the tissue is ground

in a tissue grinder. Because homogenization can destroy certain organisms (i.e., fungal elements), in some situations the tissue is minced with sterile scissors and forceps into small pieces suitable for culture.

Isolation techniques

Specimens can be inoculated to agar plates by using a general-purpose **isolation streak** to yield a semiquantitative estimate of growth. The specimen is applied by rolling the swab or placing a drop of liquid specimen onto a small area at the edge of the plate. Broth media can be inoculated by placing a few drops of the liquid specimen into the tube of broth or placing the swab into the broth. The inoculating loop is sterilized and allowed to cool thoroughly before streaking the agar. The cooled loop is passed back and forth through the inoculum in the first quadrant several times. The first quadrant should be at least one quarter of the plate, and the streak lines should be close together. The plate is turned, and quadrant two is streaked by passing the loop through the edge of the first quadrant a few times and then streaking the rest of the area. The plate is turned again, and the loop is passed a few times through the edge of quadrant two and into the rest of the third quadrant. Finally, passing the loop over the final area of the agar streaks the fourth quadrant. It may be necessary to flame the loop or turn the loop over in between quadrants, depending on the number and type of bacteria present in the specimen. Laboratory personnel must adjust their technique as necessary. When more than one agar plate is used, the loop is flamed in between plates to prevent carryover of a possible contaminant from one plate to another. The use of gamma-sterilized disposable loops eliminates the step of flaming the loop between quadrants or between plates. [Fig. 6.2](#) illustrates this technique.

The general-purpose isolation streak is useful for most specimens. The relative number of organisms can be estimated based on the extent of growth beyond the original area of inoculum. Growth only in the first quadrant can be graded as 1+, or light growth; growth in the second or third quadrant can be graded as 2+ to 3+, or moderate growth; and growth in the third or fourth quadrant can be graded as 4+, or heavy growth. Some specimens require a quantitative technique to determine the number of bacteria present. Urine specimens are inoculated using **quantitative isolation**. Plates are inoculated using a calibrated loop to deliver a specified volume. The urine is mixed well, and the calibrated loop (0.01 or 0.001 mL) is vertically inserted into the urine and transferred to the culture medium by making a single streak down the center of the plate. Without flaming, the loop is streaked back and forth through the original inoculum. [Fig. 6.3](#) illustrates this technique.

Incubation

Once the medium is inoculated, incubation conditions must be considered. The incubation conditions include both temperature and environmental atmosphere and are determined by the type of specimen and the anticipated pathogens. The laboratory processing area should contain a chart or table stipulating where each medium should be placed for incubation. Most bacteria cultures are incubated

Table 6.4 Direct Gram stain and selection of media for bacterial cultures

Specimen	Gram stain	BAP	CHOC	MAC or EMB	ANA	THIO	TM	Other
Aqueous/vitreous	X	X	X	X		X		
Blood								Blood culture bottles
Body fluids								Blood culture bottles if sufficient volume of fluid
Amniotic	X	X	X	X	X	X	X	
Bile	X	X		X	X	X		
Bone marrow	X	X	X		X	X		
Pericardial	X	X	X	X	X	X		
Peritoneal	X	X	X	X	X	X		
Pleural	X	X	X		X	X		
Synovial	X	X	X		X	X	X	
Catheter tips		X						
CSF	X	X	X			X		Cytocentrifuge recommended for Gram stain ANA culture if shunt specimen
Ear								
Inner	X	X	X	X		X		
Outer	X	X	X	X				
Eye	X	X	X	X		X		
Gastrointestinal								
Duodenal aspirate	X	X	X	X				CNA
Feces		X		X				HE or XLD, CAMPY, SMAC
Gastric aspirate	X	X	X	X				
Genital								
IUD	X					X		
Vagina/cervix	X	X	X	X			X	
Urethra	X	X	X				X	
Genital screens	W ^a							
<i>Neisseria gonorrhoeae</i>			X				X	
Group B β -hemolytic streptococci		X						Lim broth ^b
Lesion/wound/abscess	X	X	X	X	X	X		CNA if Gram stain suggests mixed gram-positive and gram-negative species
Respiratory tract: lower								
Bronchial (brush/wash/lavage)	X	X	X	X				
Sputum	X	X	X	X				Selective agar for <i>Burkholderia cepacia</i> in cystic fibrosis patients
Respiratory tract: upper								
Nasal/nasopharynx		X	X					
Sinus aspirate	X	X	X	X	X	X		
Throat		X						
Tissue	X	X	X	X	X	X		
Urine								
Catheter/void		X		X				
Suprapubic aspirate	X	X		X	X	X		

^aMale urethral specimens only.^bLim broth, selective Todd Hewitt broth with CNA.

ANA, Anaerobic culture media (anaerobic blood agar, *Bacteroides* bile esculin agar, kanamycin-vancomycin laked blood agar, anaerobic Columbia colistin–nalidixic acid agar [CNA] or phenylethyl alcohol agar); BAP, sheep blood agar; CAMPY, *Campylobacter*-selective blood agar; CHOC, chocolate agar; CSF, cerebrospinal fluid; EMB, eosin–methylene blue agar; HE, Hektoen enteric agar; IUD, intrauterine device; MAC, MacConkey agar; SMAC, sorbitol–MacConkey agar; THIO, thioglycollate broth; TM, Thayer–Martin or other *Neisseria*-selective agar; XLD, xylose–lysine–deoxycholate agar.

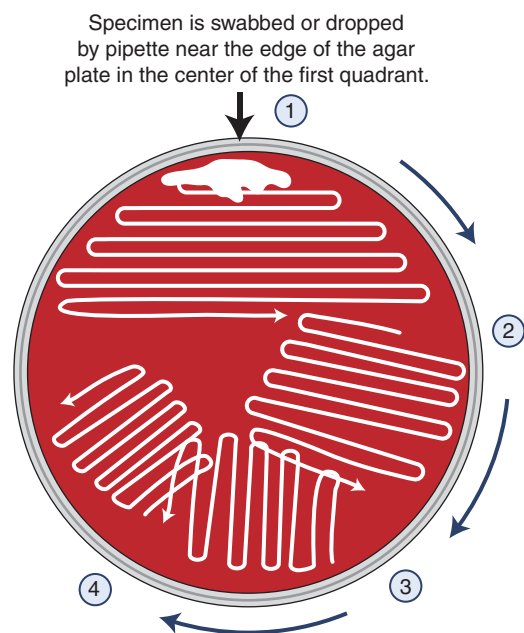


Fig. 6.2 General-purpose isolation streak.

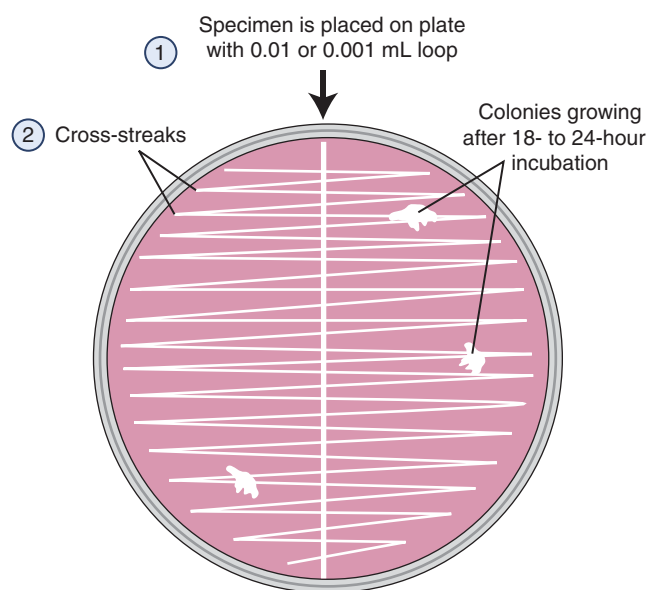


Fig. 6.3 Quantitative isolation technique.

at 35° to 37°C. Aerobes grow in ambient air, whereas **obligate anaerobes** cannot grow in the presence of oxygen and require an anaerobic atmosphere. Laboratories can achieve an anaerobic environment through the use of jars, bags, or a chamber. Some bacteria are **capnophiles** and require an increased concentration of CO₂; this can be achieved by a candle jar, a CO₂ incubator, jar, or bag. **Microaerophiles** grow with reduced oxygen and increased CO₂ and can be isolated using jars or bags. The length of incubation differs for individual organisms. Most routine bacterial cultures are held for 48 to 72 hours. Cultures for anaerobes and broth cultures may be held for 5 to 7 days. Unusual organisms may require special media or conditions beyond the routine. It

is helpful if the clinician indicates to the laboratory that an unusual organism is suspected. Table 6.5 lists unusual or fastidious organisms, recommended media, and incubation requirements.

Case check 6.3

Performing tests on specimens of poor quality would yield misleading information that could have a detrimental effect on patient care. The steps that must be taken to deal with the suboptimal specimen described in the Case in Point are as follows:

- Notify the physician to explain concerns about the specimen.
- Process the specimen because it cannot be recollected, utilizing appropriate safety measures.
- Include a disclaimer in the test report indicating the potential for compromised results.
- Document all steps/actions taken with the date, time, and who was notified.

Culture workup

The next part of the microbiology analysis is reading and interpreting the cultures. The laboratory scientist examines the culture medium and uses considerable skill and judgment in the interpretation. This interpretive judgment is the fundamental means of arriving at a result that is accurate and clinically relevant. The following questions are asked as each specimen is examined:

- What is the specimen source?
- Does this source have normal biota, or is it a sterile site?
- If normal biota is present, what bacteria are found, and what do these colonies look like?
- What are the most likely pathogens in this specimen?
- What is the colony morphology of these pathogens?
- Which medium is demonstrating growth, and what is the purpose of the medium?

This evaluation requires professional training to be able to recognize and distinguish normal biota from pathogens. The laboratory scientist must have knowledge of which organisms are pathogens in various body sites to perform a clinically relevant workup. The microscopic findings on the direct smear are incorporated into this decision-making process as they often provide a clue to the culture results.

Identification and antimicrobial susceptibility testing (when indicated) are performed on clinically relevant isolates. A challenging issue confronting laboratory scientists is the extent of the identification required. The laboratory scientist can ultimately save money by providing an accurate diagnosis in a timely fashion using a cost-effective strategy. Although definitive identification is the standard for quality patient care, microbiologists have incorporated limited identification procedures into their daily practice. These limited procedures help keep the laboratory testing cost-effective, while providing optimal patient care. Each laboratory must establish the protocol for the identification of various organisms. The introduction of newer technologies such as matrix-assisted laser desorption/ionization–time-of-flight

Table 6.5 Isolation of unusual or fastidious bacteria

Organism	Specimens	Media	Comments
<i>Bacillus anthracis</i>	Blood Skin lesion Sputum	Blood agar	Incubate at 35°–37° C, ambient air; hold plates for at least 3 days
<i>Bordetella pertussis</i>	Nasopharyngeal swab	Regan-Lowe or Bordet-Gengou agar	Bedside inoculation. Incubate at 35°–37° C, ambient air; hold plates for 6–7 days; DFA used in conjunction with culture
<i>Brucella</i> spp.	Blood Bone marrow	Commercial blood culture systems	Highly infectious by aerosol or penetration of unbroken skin; process all specimens in biosafety hood with protective equipment; incubate at 35°–37° C; most commercial systems detect growth in 7 days; conventional blood bottles should be held for 30 days and subcultured at 7, 14, and 30 days
<i>Corynebacterium diphtheriae</i>	Throat Nasopharyngeal Skin	Blood agar Loeffler agar slant Tinsdale or cystine-tellurite blood agar	Incubate at 35°–37° C, ambient air, toxigenicity testing necessary to confirm pathogenicity
<i>Francisella tularensis</i>	Eye Lymph node aspirate Skin ulcer Sputum Throat	Glucose cystine agar	Highly infectious by aerosol or penetration of unbroken skin; recommended that specimens be sent to laboratory equipped to handle them
<i>Haemophilus ducreyi</i>	Genital lesion Lymph node aspirate	Supplemented chocolate agar	Incubate at 33°–35° C, 3%–5% CO ₂ with high humidity; hold for 5 days
<i>Helicobacter pylori</i>	Gastric biopsy	BHI Brucella agar Supplemented Columbia agar	Incubate at 35°–37° C, microaerophilic atmosphere with high humidity; hold for 10 days, direct smear with silver or Giemsa stain may be diagnostic
<i>Legionella</i> spp.	Blood Lung Pleural fluid Sputum	BCYE α agar Selective BCYE α	Incubate at 35°–37° C, ambient air with high humidity; hold for at least 7 days, DFA available
<i>Nocardia</i> spp.	Brain Sputum Subcutaneous aspirate Tissue	Blood agar BHI agar SDA BCYE TM agar	Incubate at 35°–37° C, 3%–5% CO ₂ ; hold for 2–3 weeks
<i>Streptobacillus moniliformis</i>	Blood Lymph node Joint fluid	Serum-supplemented medium	Inhibited by SPS, citrate used as anticoagulant; incubate at 35°–37° C, 3%–5% CO ₂ ; hold for at least 7 days
<i>Vibrio</i> spp.	Blood Feces Lesion Tissue	Blood agar MacConkey agar TCBS agar Alkaline peptone water (enrichment for feces)	Selective agar (TCBS) needed only for fecal specimens; incubate at 35°–37° C, ambient air; hold for 3 days

BCYE, Buffered charcoal yeast extract; BCYE α , buffered charcoal yeast extract with 0.1% α -ketoglutaric acid; BHI, brain-heart infusion; DFA, direct fluorescent antibody; SDA, Sabouraud dextrose agar; SPS, sodium polyanethanol sulfonate; TCBS, thiosulfate-citrate-bile salts-sucrose; TM, Thayer-Martin.

mass spectrometry that enable definitive identification in a matter of minutes may change the approach that is taken in the workup of cultures.

Nonroutine specimens

Routine specimens have standardized processing procedures that are well established in the microbiology laboratory. However, the microbiology laboratory may be requested to process nonroutine specimens for which no processing procedures are established. In situations in which standardized procedures do not exist, the laboratory must use a standardized thought process to ensure that these specimens can be cultured in an appropriate fashion. This process begins by considering the following issues:

- Is the specimen likely to contain low numbers or high numbers of organisms? If there are low numbers of organisms, concentrating the specimen is advantageous. When few organisms are anticipated, large amounts of specimen yield better results.
- If the number of organisms is extremely low, is it important to enhance their number?
- Some specimens are being checked for sterility, and the presence of even one organism is significant. Use of a broth is important when growth must be enhanced.
- Are the organisms to be found in a specific specimen likely to be fastidious or nonfastidious? The choice of media, temperature, atmosphere, and length of incubation depends on whether the organisms are fastidious or nonfastidious.
- Is any normal biota associated with the specimen? The presence of normal biota might make special collection techniques important. It also dictates rapid transportation and timely processing.
- Does the specimen contain any preservatives or growth inhibitors that must be counteracted? Sometimes the effects of a preservative can be eliminated or reduced by dilution or use of a specific medium.
- What is a reasonable amount of specimen to culture? Are all areas of the specimen homogeneous, or will the portion chosen for culture affect the results? A 12-cm piece of vein received for culture may contain plaque on only a relatively small portion. Sampling one spot may not be representative of the whole. Sampling and grinding small pieces from multiple sites may be necessary.
- Is the objective to select a single agent from a mixed culture? An enriched or selective medium is helpful for this type of isolation.
- Is there a need to culture both external and internal surfaces? The surface to be cultured becomes important in devising beneficial methods of culturing catheters and other inanimate objects.

Nonroutine specimens may include vein grafts, multiple-lumen catheters, heart valves, implant soak solutions, perfusates, water samples, and equipment. The microbiology laboratory must establish the protocol and appropriate procedures for processing these samples. A description of the processing of some of these follows:

- *Implant soak solutions:* A large volume of soak solution and concentration is required because even one organism may be important. A broth with heavy inoculation, cytocentrifuge smear, and a large volume of filtered specimen placed on a chocolate agar plate should enable detection of low numbers of organisms.
- *Water sterility specimens:* Water from sources such as whirlpools, stills, and reagent water also requires concentration. The Millipore sampler (Millipore, Billerica, MA) is designed for this purpose and uses 18 mL of water.
- *Intrauterine devices (IUDs):* IUDs are usually cultured for the detection of *Actinomyces* spp. The device can be inoculated into a tube of thioglycollate broth.
- *Vascular catheter tips:* Vascular catheter tips are submitted for culture to aid in the diagnosis of catheter-related infection. The Maki roll technique is used in many laboratories. Using sterile forceps, a 5- to 7-cm segment of the catheter is rolled across the surface of a blood agar plate four times. After incubation, the laboratory performs identification and susceptibility tests on each organism that produces 15 or more colonies.

Case check 6.4

After cultures are incubated, the laboratory scientist evaluates the media to determine whether any potential pathogens are present. The laboratory scientist must distinguish normal biota from potential pathogens based on colony morphology. Identification of the isolate is done using stains and biochemical testing. Antimicrobial susceptibility testing will be performed on clinically relevant isolates. The culture results from the bronchoscopy specimen from the Case in Point will be evaluated and worked up according to laboratory protocol. A disclaimer will be included in the culture result indicating that the specimen was compromised during transit and the results must be interpreted with caution.

Communication of laboratory findings

The postanalytic phase of the laboratory testing process is the communication of laboratory findings. The laboratory scientist has a professional responsibility to the patient to communicate the laboratory results to the health care professional treating the patient. The laboratory must strive to provide accurate and timely information. In some situations, preliminary results are communicated as they become available. The physician can then take action on the results to provide effective patient care.

The report should clearly interpret the results and be free from microbiology jargon or abbreviations. The clinician may be unfamiliar with the laboratory procedures or the taxonomic status of the organisms involved, and it may be necessary to include interpretive statements to aid in the clinician's understanding.

Some microbiology results are considered critical values and must be reported to the physician immediately. Critical values may indicate a life-threatening situation that must be acted on promptly. The microbiology director, in consultation

BOX 6.1 Examples of critical values in microbiology

- Positive blood culture
- Positive cerebrospinal fluid Gram stain or culture
- Positive cryptococcal antigen test or culture
- Positive blood smear for malaria
- *Streptococcus pyogenes* from a sterile site
- Positive acid-fast smears or positive *Mycobacterium* culture
- *Streptococcus agalactiae* or herpes simplex virus from genital site of a pregnant woman at term
- Detection of significant pathogen (e.g., *Bordetella pertussis*, *Brucella* sp., *Legionella* sp.)

with the medical staff, should establish a list of these critical values. **Box 6.1** provides an example of critical values.

Additionally, the laboratory scientist is responsible for reporting certain infectious diseases to public health entities. It is essential that the microbiology department know which infectious agents are reportable and to which agency.

POINTS TO REMEMBER

- The microbiology laboratory must take responsibility for specimen management in the preanalytic laboratory process by ensuring that specimens are appropriately selected, collected, and transported.
- The collection of specimens for microbiology must include the use of proper technique and containers, adequate quantity, accurate labels, and prompt transportation or provisions to maintain specimen integrity.
- Shipping of clinical specimens or cultures of microorganisms must be performed according to the regulations of the DOT Hazardous Material Regulations.
- The microbiology laboratory must prioritize the processing of specimens as they are received in the laboratory based on the critical nature of the infection and the potential for specimen deterioration.
- Performing microbiology analysis on suboptimal specimens provides misleading results. The laboratory must publish guidelines for specimen rejection, and when a specimen is rejected, the laboratory must communicate this information to the person responsible for the patient.
- Macroscopic observation of the specimen allows the processor to determine the adequacy of the specimen and the need for special processing. A direct microscopic examination is useful in determining the quality of the specimen, detecting the etiologic agent, and alerting the laboratory scientist to use special procedures.
- The selection of culture media for each specimen is based on the anatomic site and the organisms likely to be involved in infection at that site. Specimens with fastidious pathogens require enriched media; specimens with an abundance of normal biota require selective media.
- If several plates are inoculated with a specimen, the media should be arranged in order, beginning with the most enriched medium and progressing to the most selective.
- The general-purpose isolation streak yields a semiquantitative estimate of growth, whereas the quantitative isolation technique will determine the number of bacteria present in a certain volume of the specimen.

- Most bacterial cultures are incubated at 35° to 37°C. The atmosphere will differ depending on the pathogens involved and may involve room air, CO₂, microaerophilic, or anaerobic conditions.
- Microbiology cultures are interpreted using skills to discriminate between normal biota and potential pathogens. The laboratory scientist must have knowledge of which organisms are pathogens in various body sites to perform a clinically relevant workup.
- The laboratory scientist performs definitive identification using accepted limited identification procedures to maintain cost-effective testing while providing optimal patient care.
- The microbiology laboratory contributes to effective patient management by communicating accurate and timely results.

LEARNING ASSESSMENT QUESTIONS

- Which one of the following involves specimen management in the preanalytic process?
 - Selecting the appropriate medium for culture
 - Performing a direct microscopic examination
 - Rejecting suboptimal specimens
 - Communicating the results of the specimen culture
- A patient has a subcutaneous infection, and the specimen is submitted on a swab. Explain why this is an unacceptable collection method. How should the sample be collected?
- Which of the following anticoagulants is appropriate for use in microbiology?
 - Citrate dextrose
 - EDTA
 - SPS
 - Sodium citrate
- Which of the following specimens should not be refrigerated?
 - Urine
 - Urogenital swab
 - Throat swab
 - Sputum
- Which of the following specimens requires immediate processing when received in the microbiology laboratory?
 - Urine
 - Cerebrospinal fluid
 - Sputum
 - Stool
- Which of the following is (are) reasons to reject a specimen for culture?
 - The specimen is preserved in formalin.
 - Information on the requisition does not match information on the specimen label.
 - A second stool sample is submitted from the same patient on the same day.
 - All of the above.
- Which one of the following is noted from a macroscopic observation?
 - White blood cells
 - Tapeworm proglottids
 - Epithelial cells
 - Protozoan cysts

8. In which of the following specimens is a direct microscopic examination not useful?
 - a. Throat swab
 - b. Sputum
 - c. Urine
 - d. Leg abscess
9. Chocolate agar is an example of which of the following?
 - a. Nonselective and differential media
 - b. Selective media
 - c. Differential media
 - d. Enriched media
10. Which of the following is an example of a selective and differential medium?
 - a. Blood agar
 - b. Chocolate agar
 - c. MacConkey agar
 - d. Modified Thayer-Martin agar
11. Which of the following specimens is cultured using a quantitative isolation technique?
 - a. Urine
 - b. Sputum
 - c. Blood
 - d. Stool
12. Cultures of *Bordetella pertussis* are incubated in ambient air at 35°C for 6 to 7 days. True or false?
13. Compose a list of questions that the laboratory scientist uses when doing a workup of a culture.
14. The microbiology laboratory receives a vascular catheter tip for culture. How should this specimen be processed?
15. What is the role of the microbiology laboratory in the postanalytic process?

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Microscopic examination of materials from infected sites

Connie R. Mahon

CHAPTER OUTLINE

Preparation of samples, 140

- Smears from swabs, 141
- Smears from thick liquids or semisolids, 141
- Smears from thick, granular, or mucoid materials, 141
- Smears from thin fluids, 141
- Cytocentrifuge preparations, 141

Stains, 142

Microscopes, 143

Terminology for direct examinations, 143

Examination of prepared material, 144

- Characterization of background materials, 146
- Search for microorganisms, 148

Grading or classifying materials, 149

- Contaminating materials, 149
- Local materials, 150
- Purulence, 151
- Mixed materials, 151

Reports of direct examinations, 151

Initiation of special handling for unsuspected or special pathogens, 152

Quality control in direct microscopic interpretations, 152

Examples of sample observations and reports, 153

Bibliography, 154

material, and purulence, and differentiate them from microorganisms that may be present in the clinical sample.

3. Describe the morphology of the microorganisms present in a direct Gram-stained smear using the descriptive terminology presented.
4. Associate the following morphology with common species:
 - Gram-negative bacilli, small, pleomorphic
 - Gram-positive cocci in clusters or chains
 - Gram-positive diplococci
 - Gram-negative diplococci
 - Gram-positive filamentous branching rods
 - Yeasts and pseudohyphae
5. Associate the direct smear results with the organisms recovered from culture.
6. Apply quality control procedures used in the microbiology laboratory to the interpretation of the direct microscopic examination and resolve any discrepancies when present.

KEY TERMS

Acid-fast

Amorphous debris

Colony-forming unit

Curschmann's spiral

Cytocentrifugation

Differential stain

Gram-negative bacteria

Gram-positive bacteria

Gram stain

Microbial morphotype

Monomicrobial

Polymicrobial

Probe-mediated stain

Purulence

Simple stain

OBJECTIVES

After reading and studying this chapter, you should be able to:

1. Given a list of stains commonly used in the medical microbiology laboratory, select the appropriate staining characteristic for determining whether a microbe is a bacterium or mycobacterium, fungus, or viral inclusion.
2. Given a photomicrograph of a Gram-stained direct smear of material from an infected site, describe the local material, contaminating

Case in point

A 75-year-old male patient with a history of chronic obstructive pulmonary disease, heavy smoking, and alcohol abuse came to his physician with a fever, chills, and a productive cough. Sputum samples were sent to the laboratory for direct smear and culture. Blood cultures also were drawn three times within 24 hours of hospital admission. The direct smear was

Gram-stained (Fig. 7.1). The sputum culture produced a heavy growth of α -hemolytic colonies. Blood cultures yielded similar results after 24 hours of incubation.

Issues to consider

After reading the patient's case history, consider:

- The role of the Gram stain and microscopic morphology in identification of microorganisms
- The presence of purulence (inflammation) in the direct smear of the specimen and its significance
- The evidence of contamination by normal (resident) microbiota (flora) and what it indicates
- The significance of the suspected organism observed—if it is “real” or an artifact
- The results for the microorganisms used as quality control (QC) to determine whether the Gram stain procedure was performed properly on the direct smear
- The stained appearance of the elements present (e.g., polymorphonuclear cells, red blood cells, and epithelial cells) to ensure that their appearance is correct

Differential staining and microscopy provide the foundation of the laboratory diagnosis of infectious diseases. Direct microscopy, visualization of microorganisms in clinical specimens, has been possible for more than 200 years. However, it was not a practical reality until Koch established the germ theory of disease in the 1880s. By 1880, a Scottish surgeon had published his direct observations of cluster-forming cocci in **purulence** from human disease. He named these cocci *Staphylococcus*. In 1884, Christian Gram developed the **Gram stain**, which places most bacteria into one of two groups: **gram-positive bacteria** or **gram-negative bacteria**. Since then, the Gram stain has allowed microbiologists to examine a clinical specimen directly for microorganisms such as the gram-positive coccus *Staphylococcus*. There are now several stains available to help microbiologists visualize microorganisms in clinical samples. Because of their small size, viruses cannot generally be detected by staining.

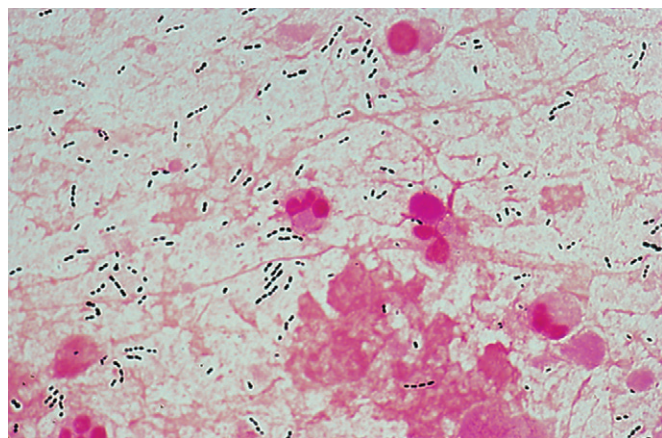


Fig. 7.1 Expecterated sputum smear, Gram stain, light microscopy (medium-power view). Purulence, light. Amorphous debris, moderate. Gram-positive diplococci, encapsulated, extracellular.

However, the morphologic changes some viruses cause in infected cells can be seen and might aid in the identification of a viral infection. These changes are called *cytopathic effect* (see Chapter 29).

In many instances, the physician has a correct idea about the diagnosis after taking a patient history and performing a physical examination. In other instances, in which the diagnosis is not evident, assistance comes from laboratory or radiologic studies. With infectious diseases, the physician might have an idea of the likely etiology from the rate of symptom progression and is able to evaluate the extent of the infectious process. Nevertheless, specimens are collected and sent to the laboratory to confirm the physician's suspicions about the patient's illness. Direct viewing of pathogens becomes primary or direct evidence to confirm or refute the physician's initial clinical impression. Hence, the ability of the laboratory to respond quickly to the physician with useful results is key to appropriate treatment or changing treatment if the physician's presumptive diagnosis was incorrect. The diagnostic microbiology laboratory has the opportunity to respond to the physician during the treatment decision-making process or early in presumptive therapy. If this impression is incorrect, reconsideration is facilitated, and additional studies can be undertaken as needed. Culture results usually are received too late to alter presumptive therapy. At best, they confirm the correctness of the therapeutic choices already made and implemented.

Preparation of samples

Samples for routine bright-field microscopy are prepared in a manner that facilitates adequate examination within a reasonable time. For smears, specimens should be examined grossly to determine the best approach (Table 7.1). Both thick, but not opaque, and thin (monolayer) smear areas should be produced by the smear process chosen.

Table 7.1 Preparing infected materials for visual examination

Preparation	Specimen or organism type
For gross examination	
Wet preparation	Parasites Materials >1 mm in size
For microscopic examination	
Wet preparation (direct or sedimented)	Fluids or semisolids
Cytoцентрифугед (direct or presedimented)	Clear or slightly turbid fluids
Smear	Clear or slightly turbid fluids
1. Drop	Pus Tissue homogenate
2. Pellet, centrifuged	Blood culture Dilute specimen
3. Rolled swab	Swabbed material
4. Imprint (touch preparation)	Tissue

Smears from swabs

Smears from swabs are prepared by rolling the swab back and forth over contiguous areas of the glass slide to deposit a thin layer of sample material (Fig. 7.2). This process preserves the morphology and relationships of the microorganisms and cellular elements. In addition, the swab should not be rubbed back and forth across the slide because important material on the opposite side of the swab might not be deposited, and cellular elements could be broken up. If the sample can be collected only on swabs, ideally two swabs are submitted. Smears should not be prepared from a swab after it has been used to inoculate culture media.

Smears from thick liquids or semisolids

Swabs also can be used for preparation of smears from thick liquid or semisolid specimens such as feces (Fig. 7.3). The swab is immersed in the specimen for several seconds and used to prepare a thin spread of material on the glass slide for staining and viewing. This swab method of preparation is adequate but may produce less desirable results than other methods.

Smears from thick, granular, or mucoid materials

Opaque material must be thinly spread so a monolayer of material is deposited in some areas. It is most desirable to have both thick and thin areas. Granules within the material must be crushed so their makeup can be assessed. A better

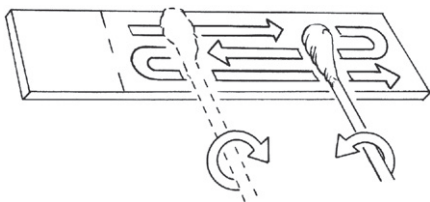


Fig. 7.2 Smear preparation of a sample collected on a swab. The swab should be rolled back and forth across the slide to deposit the sample completely.

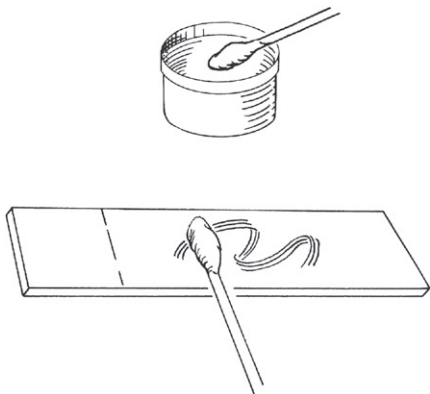


Fig. 7.3 Smears from opaque thick liquids or semisolids, such as stool, can be made with a swab to sample and smear the material.

presentation of granules is possible if granules or grains are “fished” from the surrounding materials and crushed on a separate slide using the technique shown in Fig. 7.4. Granules that are too hard to crush between two glass slides probably do not represent infectious materials. More likely, they are small stones or foreign bodies. Examination using a dissecting microscope may help characterize the nature of hard granules.

Steps to prepare a smear from thick, granular, or mucoid materials are as follows:

1. Place a portion of the sample on the labeled slide, and press a second slide, with the label down, onto the sample to flatten or crush the components.
2. Rotate the two glass surfaces against each other so the shear forces break up the material.
3. Once the material has been flattened and sufficiently thinned, pull the glass slides smoothly away from each other to produce two smears.
4. If the material is still too thick, repeat the first three steps with another (third) glass slide. The best smear or both smears can be retained for staining.

Slides of material from difficult sample sites, scant samples, or patients with critical illnesses should not be discarded until the culture evaluation is complete.

Smears from thin fluids

Thin fluids such as urine, cerebrospinal fluid (CSF), and transudates can be prepared by drawing up a small quantity of the fluid or a resuspended sediment of the fluid into a pipette and depositing it as a drop of fluid onto a clearly marked area on the slide (Fig. 7.5). “Thin” specimens of fluids should not be spread on the slide. The area of sample drop should be marked on the reverse side of the slide using a wax pencil or placed within the circle or well of a premarked slide. If available, **cytocentrifugation** should be used to prepare this type of specimen. The material, after staining, may not be grossly visible because of a low protein or cell count.

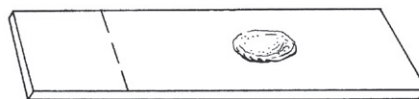
Cytocentrifuge preparations

Cytocentrifugation is an excellent method for preparing non-viscous fluids such as CSF and bronchoalveolar lavage fluids. In the cytocentrifugation process, cellular elements and microorganisms from the specimen are deposited within a discrete area onto the surface of a glass slide as a monolayer for easy viewing (Fig. 7.6A). Protein, which stains gram-negative, is dissipated into a filter pad, leaving the background clearer for viewing gram-negative morphotypes. Cell morphology is good, and the concentrating effect shortens viewing time and increases the volume of cellular material reviewed.

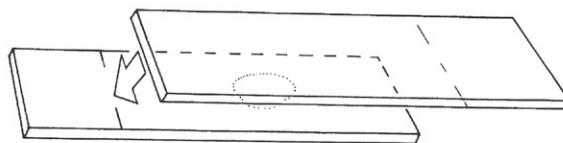
Cytocentrifuge technique

A cytocentrifuge with a closed bowl is preferred for microbiology. The bowl can be loaded and unloaded within a biological safety cabinet to avoid possible infectious aerosols. The steps of the technique are as follows:

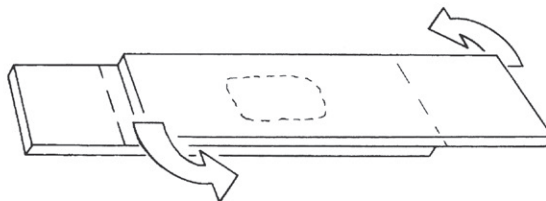
1. Small aliquots of fluid (0.1 to 0.2 mL) are placed in the cytocentrifuge holders.



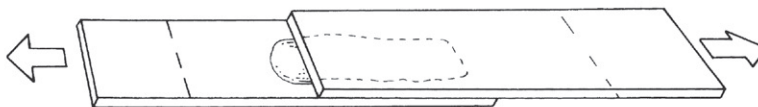
Drop or place the sample onto the surface of a labeled glass slide.



Place the second slide face down over the material.



Press to flatten or crush the material, and rotate the two glass surfaces against each other.



Pull to spread.

Fig. 7.4 Preparation of smears from thick, granular, or mucoid samples.

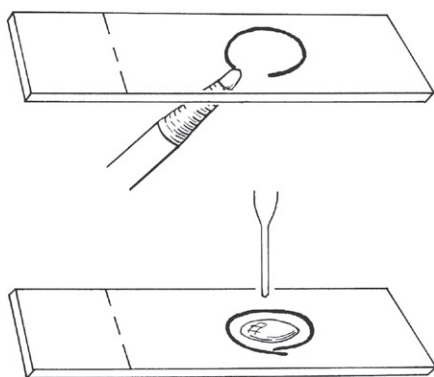
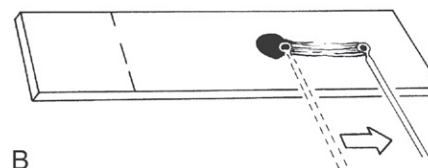


Fig. 7.5 Smears from thin fluids can be prepared by placing a single drop of fluid or resuspended fluid sediment on a well-marked area of the slide.



A



B

Fig. 7.6 **A**, Cytocentrifuge preparations deposit the concentrated sample within a limited area for viewing. **B**, If the deposit is too heavy, a portion of the material may be smeared to produce a thin area.

2. The material is spun for 10 minutes.
3. The slide is removed. If the deposit of cells is too heavy, a portion of the cellular deposition can be smeared (see [Fig. 7.6B](#)).
4. The sediment is fixed and decontaminated in 70% alcohol for 5 minutes.

Stains

Staining imparts an artificial coloration to the smear materials that allows them to be seen using the magnification provided by a microscope. There are many types of stains: **simple stains**,

differential stains, and **probe-mediated stains**. Simple stains are directed toward coloring the forms and shapes present, differential stains are designed to color specific components of the elements present, and antibody or DNA probe-mediated stains are directed specifically at identification of an organism or virus. Some stains, such as India ink on spinal fluid, are used as wet mounts on liquid specimens. This is a differential stain allowing the detection of the encapsulated yeast *Cryptococcus neoformans*. Stains most commonly used in the diagnostic microbiology laboratory are listed in [Table 7.2](#). Four stains—Gram, **acid-fast**, calcofluor white, and rapid modified Wright-Giemsa—should be available in all diagnostic microbiology laboratories (see procedures in [Appendix D on Evolve](#)). Most other stains are directed toward specific organism groups and should be available when needed.

Microscopes

In most circumstances, the requested tests provide a guide to the examination of clinical specimens submitted for microbiologic studies. A routine approach to specimen management, however, should include an examination procedure that would discover unexpected pathogens. In most diagnostic

microbiology laboratories, this procedure consists of performing a gross visual inspection at the time of smear and culture preparation and then proceeding to the level of magnification needed to identify the pathogen or determine that no pathogen is present. The purpose of microscopic examination of a Gram-stained sample is to look for structures too small to be seen with the unaided eye.

Microscopes differ both in their ability to resolve small structures and in their modifications. Microscopes are divided into two basic types: compound light microscopes, with common resolving limits of 1 to 10 μm and enlargements up to $\times 1000$, and electron microscopes, with enlargements greater than $\times 1,000,000$ ([Table 7.3](#)). The microbiology laboratory uses several modifications of the compound light microscope, but the “workhorse” of the laboratory is the bright-field microscope.

Terminology for direct examinations

The microscopist must have a consistent vocabulary for the description of materials seen in samples. This vocabulary must be shared by the microbiology and medical communities so when observations are reported, everyone understands

Table 7.2 Stains for infected materials

Stains	Applications
General morphology	
Wright-Giemsa	Bronchoalveolar lavages Tzanck preparations Samples with complex cellular backgrounds (visualizes bacteria, yeasts, parasites, and viral inclusions)
Selected morphology	
Leifson	Flagella
Methylene blue	Metachromatic granules of <i>Corynebacterium diphtheriae</i>
Acid-fast stains	
Ziehl-Neelsen	Direct smears for mycobacteria in material from the lower respiratory tract Sediments for mycobacteria (concentrated smears) Partial acid-fastness of <i>Nocardia</i> spp.
Kinyoun	Direct smears for mycobacteria in material from the lower respiratory tract Sediments for mycobacteria (concentrated smears) Acid-fast stain modification of Ziehl-Neelsen method for <i>Cryptosporidium</i> and <i>Cyclospora</i> parasites in stool specimens
Fluorochrome	Direct smears for mycobacteria in material from the lower respiratory tract Sediments for mycobacteria (concentrated smear, auramine and rhodamine) Preferred acid-fast stain to detect acid-fast organisms
Calcofluor white	Bronchoalveolar fungi and some parasitic cysts Differentiates them from background materials of similar morphology
Gram stain	
Traditional	Routine stain for detecting microorganisms Yeasts differentiated from all other organisms
Enhanced	Provides same differential staining but enhances red-negative organisms by staining the background material green to gray-green
Genus (or species)-specific stains	
Antibody or DNA probe stains	Used for specific identification of selected pathogens, such as <i>Chlamydia trachomatis</i> , <i>Bordetella pertussis</i> , <i>Legionella pneumophila</i> , herpes simplex virus, varicella-zoster virus, cytomegalovirus, adenovirus, and respiratory viruses

Table 7.3 Observing microbial pathogens

Tools	Magnification (×)	Application
Unaided eyes	0	Gross examination
Magnifying glass	5	Gross examination
Dissecting microscope	2.5–30	Gross detailed examination and manipulation
Compound light microscope		
Bright-field	10–1000	Cells stained
Dark-field	10–400	Cells not readily stained for bright-field microscopy
Phase-contrast	10–400	Living or unstained cells
Fluorescence	10–400	Preparations using fluorochrome stains, which can directly stain cells or be conjugated to antibodies that attach to cells
Electron microscopes		
Transmission electron	150–2 million	Determine ultrastructure of cell organelles Visualize virus particles
Scanning electron	20–10,000	Determine cellular surface shapes and structures

the implications of the descriptions. Common observations can be coded so they are consistent among observers. The use of computers for recording coded observations and generating reports of the findings extends the need for uniform terminology further. Only unusual findings should be described individually in a report.

The background of the sample being evaluated should be described in sufficient detail to convey the composition of the material. The presence of cells representing a response to injury supports the probability of infection and directs attention toward specific types of pathogens. Common morphotype descriptions and the most prevalent associated species are listed in Table 7.4. Examples of useful descriptive phrases with quantitation are listed in Table 7.5.

Microorganisms can be described in such a way that, based on prevalence, the description implies the identification of the organism. For example, the observation of a gram-negative pleomorphic coccobacillus from the spinal fluid of a child implies that *Haemophilus influenzae* is the infecting agent.

Examination of prepared material

A limited number of microbial pathogens from commonly sampled infected sites are regularly encountered by the microbiologist. The Gram stain and acid-fast stain are the fastest and least expensive methods for presumptive diagnosis in common clinical settings. Organisms are readily seen

Table 7.4 Gram stain morphology and associated organisms

Morphotype description	Most common organisms
Bacteria	
Cocci	
Gram-positive cocci	<i>Aerococcus</i> , <i>Enterococcus</i> , <i>Finegoldia</i> , <i>Leuconostoc</i> , <i>Pediococcus</i> , <i>Planococcus</i> , <i>Staphylococcus</i> , <i>Stomatococcus</i> , <i>Streptococcus</i>
Gram-positive cocci	
Pairs	<i>Finegoldia</i> , <i>Staphylococcus</i> , <i>Streptococcus</i> , <i>Enterococcus</i> spp.
Tetrads	<i>Micrococcus</i> , <i>Staphylococcus</i> , <i>Peptostreptococcus</i> spp.
Groups	<i>Finegoldia</i> , <i>Staphylococcus</i> , <i>Peptostreptococcus</i> , <i>Stomatococcus</i> spp.
Chains	<i>Streptococcus</i> , <i>Peptostreptococcus</i> spp.
Clusters, intracellular	Microaerophilic <i>Streptococcus</i> spp., viridans streptococci, <i>Staphylococcus</i> spp.
Encapsulated	<i>Streptococcus pneumoniae</i> , <i>Streptococcus pyogenes</i> (rarely), <i>Stomatococcus mucilaginosus</i>
Gram-positive diplococci (lancet-shaped)	<i>Streptococcus pneumoniae</i> , often encapsulated
Gram-negative diplococci	<i>Pathogenic Neisseria</i> spp., <i>Moraxella catarrhalis</i>
Bacilli	
Gram-positive bacilli	
Small	<i>Listeria monocytogenes</i> , <i>Corynebacterium</i> spp.
Medium	<i>Lactobacillus</i> , anaerobic bacilli
Large	<i>Clostridium</i> , <i>Bacillus</i>
Diphtheroid	<i>Corynebacterium</i> , <i>Cutibacterium</i> , <i>Propionibacterium</i> , <i>Rothia</i> spp.
Pleomorphic, Gram-variable	<i>Gardnerella vaginalis</i>
Beaded	Mycobacteria, antimicrobial-affected lactobacilli, and corynebacteria

Table 7.4 Gram stain morphology and associated organisms—cont'd

Morphotype description	Most common prganisms
Filamentous	Anaerobic morphotypes, antimicrobial-affected cells
Filamentous, beaded, branched	<i>Actinomycetes</i> , <i>Nocardia</i> , <i>Nocardiosis</i> , <i>Streptomyces</i> , <i>Rothia</i>
Bifid or V forms	<i>Bifidobacterium</i> , <i>brevibacteria</i>
Gram-negative coccobacilli	<i>Bordetella</i> , <i>Haemophilus</i> (pleomorphic)
Masses	<i>Veillonella</i>
Chains	<i>Prevotella</i> , <i>Veillonella</i>
Gram-negative bacilli	
Small	<i>Haemophilus</i> , <i>Legionella</i> (thin with filaments), <i>Actinobacillus</i> , <i>Bordetella</i> , <i>Brucella</i> , <i>Francisella</i> , <i>Pasteurella</i> , <i>Capnocytophaga</i> , <i>Prevotella</i> , <i>Eikenella</i>
Bipolar	<i>Klebsiella pneumoniae</i> , <i>Pasteurella</i> , <i>Yersinia</i> , <i>Bacteroides</i>
Medium	Enterics, pseudomonads
Large	Devitalized clostridia or bacilli
Curved	<i>Vibrio</i> , <i>Campylobacter</i>
Spiral	<i>Campylobacter</i> , <i>Helicobacter</i> , <i>Gastrobacillum</i> , <i>Borrelia</i> , <i>Leptospira</i> , <i>Treponema</i>
Fusiform	<i>Fusobacterium nucleatum</i>
Filaments	<i>Fusobacterium necrophorum</i> (pleomorphic)
Yeasts and fungi	
Yeasts	
Small	<i>Histoplasma</i> , <i>Torulopsis</i>
Medium	<i>Candida</i>
With capsules	<i>Cryptococcus neoformans</i> and <i>Cryptococcus gatti</i>
Thick-walled, broad-based bud	<i>Blastomyces</i>
Hyphae	
Septate	Fungi
Aseptate (sparsely septate)	Zygomycetes
With arthroconidia	<i>Coccidioides</i>
With branches at a 45-degree angle	<i>Aspergillus</i>
Pseudohyphae	<i>Candida</i>
Spherule with endospores	<i>Coccidioides</i>
Sporangia with endospores	<i>Protothecae</i>

Table 7.5 Descriptions of background material

Cells and structures	Associations
Amorphous debris (light, moderate, or heavy)	Necrosis, heavy protein fluid
Black particulate debris	Smoke inhalation, crack cocaine
Charcot-Leyden crystals	Eosinophils
Curschmann's spirals (sputum)	Bronchospasm, obstruction, asthma
Epithelial cells (light, moderate, or heavy)	Epithelial surface involved or adjacent to collection site
Epithelial with contaminating bacteria	Passage of specimen through contaminated area during collection
Intracellular organisms	Inferred association in infection
Local material (light, moderate, or heavy)	Reflection of collection site
Mononuclear cells present	Chronic inflammation
Mucus (light, moderate, or heavy)	Irritation of glandular surface
Purulence (none, light, moderate, or heavy)	Acute inflammation, exudation
Red blood cells	Trauma, hemorrhage

because more than 10^5 **colony-forming units** of infecting organisms per milliliter are commonly present in clinically evident infections.

Two types of infection are important to distinguish: infections caused by a *single species*, or **monomicrobial**, and infections caused by *multiple species*, or **polymicrobial**. The single agents of infection or agents causing classic infections are easily recognized by microscopy and require limited interpretations. These agents and infections include *Streptococcus pneumoniae* pneumonia, *Staphylococcus aureus* abscesses or pyodermas, *H. influenzae* tracheobronchitis or meningitis in children, *Clostridium perfringens* gas gangrene, *Nocardia* spp. lung abscesses, and gonococcal urethritis in males (Fig. 7.7).

Polymicrobial presentations in smears require further interpretation and must consider the smear background, morphology of the organisms, and the anatomic location of the suspected infection as well as accompanying clinical symptoms. Polymicrobial infections usually arise from displaced normal or altered microbiota, and culture yields the same species that can be isolated in culture from uninfected but contaminated specimens. These infections usually represent displacement of environmental, skin, oropharyngeal, gastrointestinal, or vaginal biota into tissues, with subsequent infection. Commonly encountered infections of this type are surgical wound (skin biota) infection, aspiration (oropharyngeal biota) pneumonia, perirectal (fecal biota) abscesses, and tubo-ovarian (vaginal biota) abscess (Fig. 7.8).

Case check 7.1

The Gram-stained smear of the expectorated sputum sample taken from the patient described in the Case in Point (see Fig. 7.1) showed moderate, **amorphous debris** and extracellular, encapsulated, gram-positive diplococci. This is a typical smear presentation for early pneumococcal pneumonia. The pneumococci have proliferated to high numbers, and the lung is responding with increased mucus and fluid release. There is early migration of neutrophils, but phagocytosis of the diplococci is limited. Routine bacterial culture of this sample should yield a heavy growth of *S. pneumoniae*.

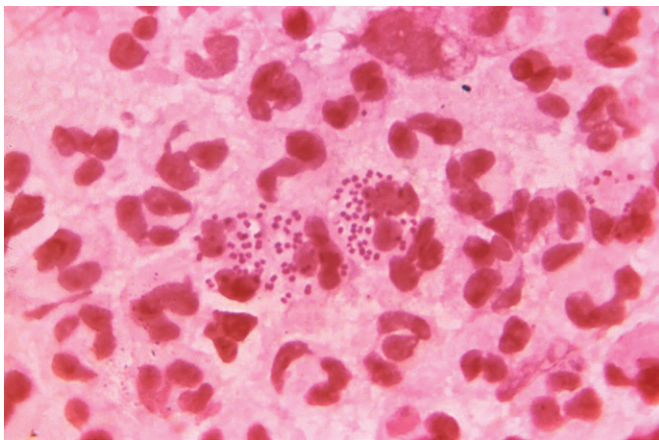


Fig. 7.7 Urethral exudate, smear, Gram stain light microscopy human papillomavirus (HPV). Purulence. Intracellular, gram-negative, diplococci consistent with *Neisseria gonorrhoeae* among numerous white blood cells known as *polymorphonuclear (PMN) leukocytes*. (Courtesy Bill Schwartz, Centers for Disease Control and Prevention, Public Health Image Library, Atlanta, GA.)

Characterization of background materials

The laboratory scientist should look at the slide material with the unaided eye before beginning the microscopic examination. The distribution and consistency of the material should be noted. The microscope's low-power objective ($\times 2.5$ to $\times 10$) should be used first to evaluate the general content of the material on the slide. Specimens can be homogeneous or heterogeneous and may contain pathogens evenly distributed throughout the specimen or limited to one visual field. A mental inquiry checklist should be followed until the habit of searching a slide systematically is developed. Items that should be included in such a checklist include:

- *Is there evidence of contamination by normal (resident) microbiota?* The laboratory scientist should look for squamous epithelial cells (SECs), bacteria without the cells of inflammation, food, or other debris. Does this material constitute the entire sample, or is there a representative sample also available in a manner that can be recognized? Contamination of specimens not collected from sterile sites diminishes the value of culture studies.
- *Is necrotic (amorphous) debris in the background?* Infection with organisms such as *C. perfringens* and *Nocardia* spp. may elicit few polymorphonuclear (PMN) leukocytes. The inflammatory cells that migrate into the area of infection can be lysed (Fig. 7.9). Patients with leukopenia also may have few inflammatory cells within their inflammatory debris. Amorphous debris usually is the remains of tissue mixed with the breakdown products or fluids of acute inflammation and always should be searched for organisms. *Mycobacterium tuberculosis* organisms stain poorly as beaded gram-positive bacilli or not at all with Gram stain (Fig. 7.10); they can appear within necrotic debris as negative images (Fig. 7.11).
- *Is there evidence of purulence?* Purulence with red blood cells, neutrophils, protein background, and necrosis reflects acute inflammation (Fig. 7.12). The image shows an abscess aspirate with the presence of heavy purulence. Mononuclear cells, including lymphocytes, monocytes,

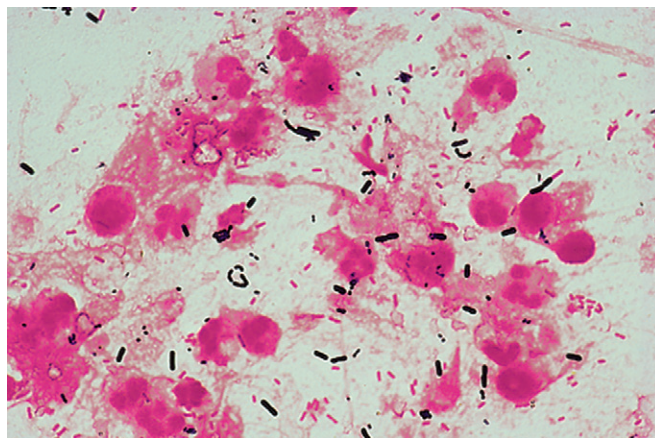


Fig. 7.8 Aspirated sputum, smear, Gram stain, light microscopy (medium-power view). Purulence, light. Local materials, light. Gram-positive bacilli, large. Gram-negative bacilli, medium, intracellular. Gram-positive cocci, pairs, chains. Morphology suggests polymicrobial infection, aspiration pneumonia.

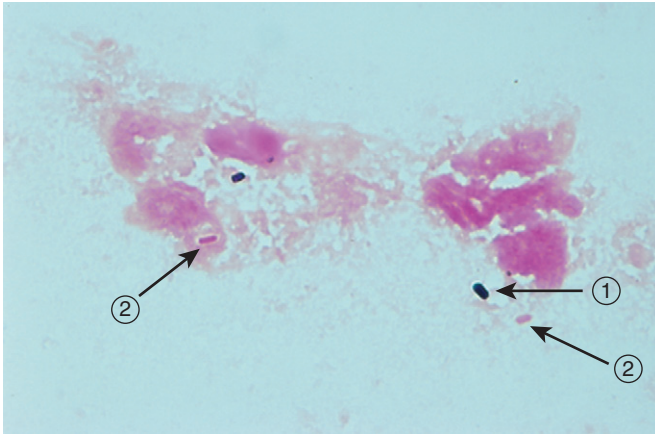


Fig. 7.9 Wound cellulitis, smear, Gram stain (medium-power view). Purulence, none. Amorphous debris, moderate. Gram-positive bacilli, large. Gram-negative bacilli, large. Morphology consistent with *Clostridium* spp. *Impression*: gas gangrene. The growth rate of this organism is rapid, and both viable (gram-positive, arrow 1) and nonviable (gram-negative) bacilli can be present in the smear material (arrows 2).

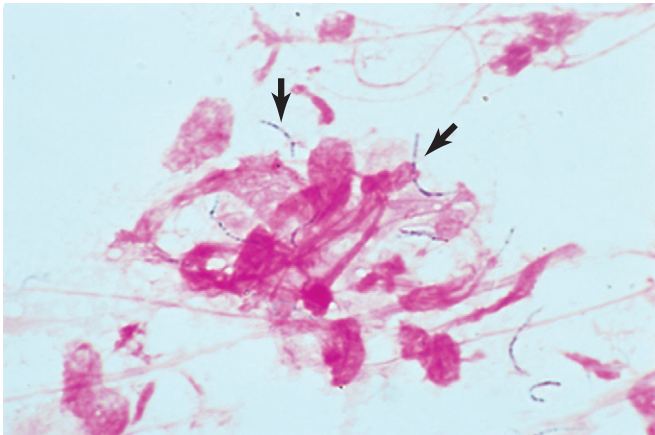


Fig. 7.10 Abscess aspirate, smear, Gram stain, light microscopy (high-power view). Purulence, moderate. Amorphous debris, light. Gram-positive bacilli, beaded (arrows). Suspect mycobacteria: initiate additional testing (see Plate 19).

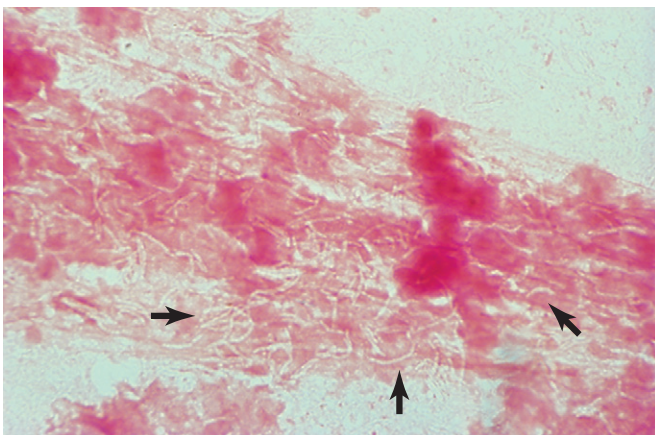


Fig. 7.11 Expecterated sputum, smear, Gram stain, light microscopy (medium-power view). Amorphous debris, heavy. Bacillary shapes, negative image (arrows). Oil removed from smear, decolorized with acid alcohol, and immediately restained with Ziehl-Neelsen acid-fast stain. Acid-fast bacilli, numerous. Specimen recovered; acid-fast culture requested by laboratory. Suspicious for tuberculosis.

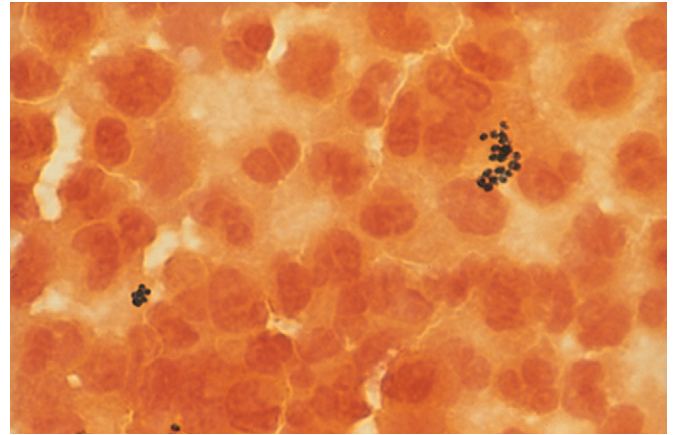


Fig. 7.12 Abscess aspirate, smear, Gram stain, light microscopy (medium-power view). Purulence, heavy. Gram-positive cocci, groups, extracellular. *Impression*: staphylococcal disease. Aerobic and anaerobic culture plates were negative at 24 hours. There was culture isolation of *Staphylococcus aureus* from this same abscess 5 days previously. The patient was being treated with clindamycin. Because the cocci in the smear did not appear to be damaged by the antimicrobial agent, another species of gram-positive coccus was sought. The anaerobic culture grew *Peptostreptococcus* sp., which is clindamycin resistant.

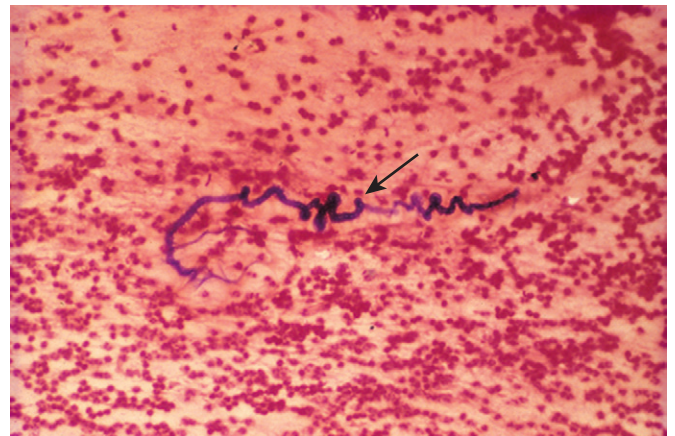


Fig. 7.13 Expecterated sputum, smear, Gram stain, light microscopy (medium-power view). Purulence, heavy. Local materials, Curschmann's spiral. No organisms seen. The Curschmann's spiral (arrow) is material local to the tracheobronchial tree but is not normal, so it is specifically reported. This spiral may manifest in various sizes depending on the size of the bronchus involved. Spirals can be particularly prominent after an asthmatic episode with bronchial constriction.

and macrophages, reflect chronic inflammation. Patients who are cytopenic do not have the cellular response seen in normal individuals. Purulence, blood, and necrosis can be present if traumatic tissue damage occurs in the absence of infection.

- *Are unexpected structures present?* The characteristic coiled structure of a **Curschmann's spiral** (Fig. 7.13), a mucus plug found in sputum of patients with asthma, is more easily recognized on low power. This structure must not be confused with parasitic larvae, which also can be found in sputum using low-power magnification. Large granules, grains, or fungal forms such as spherules or fungal mats can best be recognized at low power.

Search for microorganisms

After the full extent of the material has been examined on low power, a representative area should be selected for viewing with higher magnification. A $\times 40$ or $\times 60$ objective lens is preferred for scanning, and a $\times 100$ oil immersion lens is used for final evaluation. In infection, the organisms will be intimate with the purulence of necrotic debris. All grains or granules and the background should be examined carefully. The delicate gram-positive filaments of *Nocardia* spp. can blend into the background (Fig. 7.14). *H. influenzae* may be present in large numbers, hidden within the mucus, in acute exacerbations of chronic bronchitis (Fig. 7.15). Intracellular and extracellular forms should be noted. Strict criteria for **microbial morphotypes** should be maintained. The examiner must not be distracted by precipitated gram-positive stain, keratohyalin granules (Fig. 7.16) or other artifacts. Organisms should be evaluated for shape, size, and Gram stain reaction. Because cell wall-damaged bacteria, antimicrobial-treated bacteria,

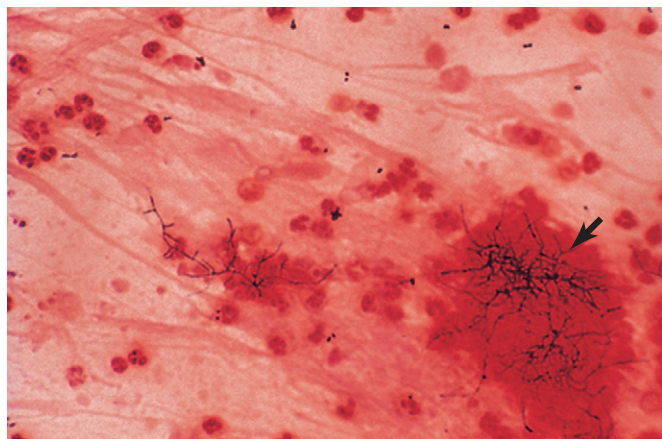


Fig. 7.14 Expectorated sputum, smear, Gram stain, light microscopy (high-power view). Purulence, heavy. Local materials, moderate. Grain present. Gram-positive bacilli, filamentous, beaded, branched (arrow). Suspicious for *Nocardia* or *Actinomyces* (see Plate 23).

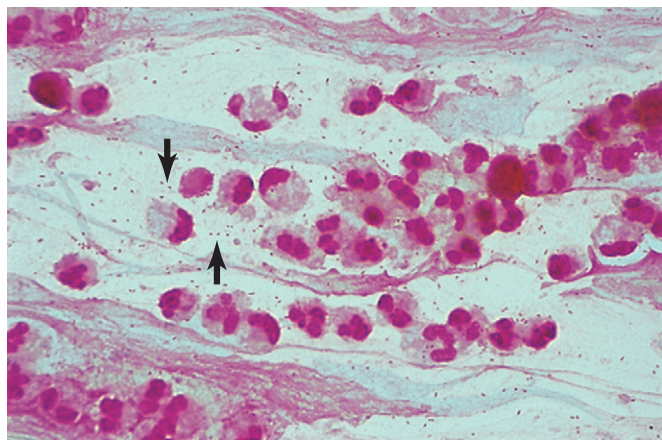


Fig. 7.15 Expectorated sputum, smear, Gram stain, light microscopy (high-power view). Purulence, moderate. Local materials, heavy. Mucus present. Gram-negative coccobacilli, intracellular, extracellular (arrows). Morphology consistent with *Haemophilus influenzae*.

or dead bacteria can appear falsely gram-negative, their shapes and sizes are critical “co-characteristics.” The classic misleading smear example is the observation of gram-negative, lancet-shaped diplococci mixed with the predominant gram-positive forms. An inexperienced observer might misinterpret this as mixed infection rather than as the simple presence of dead pneumococci in a classic infection. Further considerations include:

- *Is there a single most probable etiologic microorganism?* If so, is the morphology sufficiently characteristic to presume identification? Is there a specified antimicrobial drug for treatment of infections with this infectious agent? Is antimicrobial susceptibility testing necessary, or is identification of the suspected pathogen sufficient for empiric treatment?
- *Is the infection monomicrobial or polymicrobial?* Do morphotypes present characterize the source of the organisms? Can the mixture of organisms be characterized?
- *Examine more than one area of the smear.* More than one microorganism should be found if possible. It is rare not to be able to find more than one cell, because in an infection organisms are usually distributed throughout the specimen. Care should be taken in the interpretation of very low numbers of bacteria, especially in the absence of inflammation or necrosis and in specimens from non-sterile sites. Small numbers of organisms in samples from sterile sites must be seriously considered. However, additional smears can be made and examined if the likelihood of contamination is high.
- *Do not overinterpret the findings.* Specific diagnosis should be limited to a small number of instances in which the smear is classic in its presentation and the extent of infection is not an issue. If acid-fast bacteria are suspected, an acid-fast stain should be performed before an opinion is rendered. If a fungal element is suspected, a calcofluor white stain should be performed. Both of these follow-up stains can be performed on the decolorized, Gram-stained preparation.

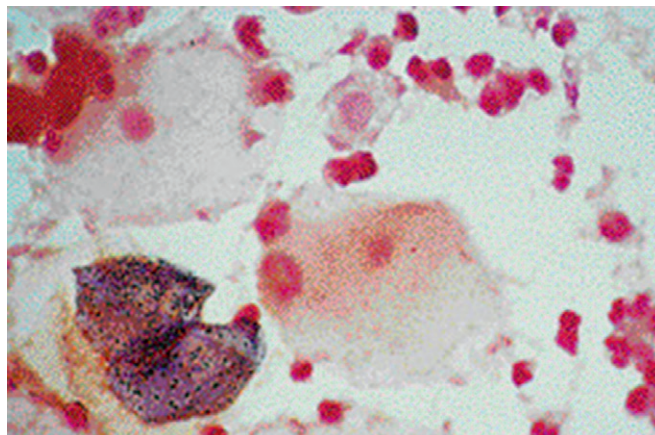


Fig. 7.16 Amniotic fluid, cytocentrifuge preparation, Gram stain, light microscopy, medium-power view. Purulence, moderate. Local materials, moderate. No organisms seen. The presence of purulence (neutrophils or polymorphonuclear [PMN] leukocytes) indicates a process suspicious for infection. The absence of organisms in this normally sterile fluid is a critical observation. Squamous epithelial cells are local to this specimen type and confirm that the sample is amniotic fluid. The blue keratohyalin granules must not be mistaken for bacteria.

Grading or classifying materials

Microscopic examination can also immediately reveal that a specimen is unlikely to be helpful in diagnosis or culture management. The specimen may be just blood rather than infected material, or it may be oropharyngeal surface debris or some other normal surface material. Processing and culture interpretation of such nonrepresentative specimens may lead to delayed treatment because of a false-negative culture or to inappropriate antimicrobial therapy because of a false-positive culture from growth of normal biota or antimicrobial-altered biota.

Several grading or classification systems have been derived to aid the laboratory scientist in arriving at decisions relating to culturing the specimen or interpretation of growth from culture of a specimen. Most evaluations are aimed at specimens such as sputum, for which collection is complicated by contamination with throat and mouth biota because culture alone can be misleading. The objective is to separate the representative sample from the contaminated sample before culture or culture evaluation. Bartlett's Q scoring of sputum samples (Table 7.6) and the Murray-Washington method (Table 7.7) for contamination assessment document the association of 10 to 20 SECs per microscopic field at $\times 100$ magnification with unacceptable specimens and 10 to 25 PMN leukocytes per field at $\times 100$ magnification with significant specimens. Heineman's method emphasizes the ratio between SECs and PMN leukocytes.

In some cases, direct smear examination is a means for diagnosis. The Nugent scoring system for Gram-stained vaginal smears (see Table 16.4) is used to diagnose bacterial vaginosis. Stained smears are examined and scored for the presence of *Lactobacillus*, *Gardnerella*, and *Mobiluncus*

Table 7.6 Grading the quality of the clinical sample using Bartlett's Q score

Score	Average number of neutrophils per low-power field	Score	Average number of squamous epithelial cells per low-power field
0	0 (none)	0	0 (none)
+1	1–9 (few)	–1	1–9 (few)
+2	10–24 (moderate numbers)	–2	10–24 (moderate numbers)
+3	≥ 25 (many, numerous)	–3	≥ 25 (many, numerous)

Interpretation:
 Q score = (points for average number of neutrophils) + (points for average number of squamous cells)
 Minimum score = –3
 Maximum score = +3
 The higher the score, the better quality the specimen.

- A specimen with a composite score $\geq +1$ should be cultured.
- A sputum specimen from a leukopenic patient with ciliated respiratory epithelial cells should be cultured.
- A composite Q score that is 0 or negative is probably a superficial sample that may not be a reliable specimen and so is usually not cultured.

From Bartlett, R. C. (1974). *Medical microbiology: quality, costs and clinical relevance*. Malden, MA: John Wiley & Sons.

morphotypes. Observation of Gram-stained smears is more accurate than cultures in diagnosing bacterial vaginosis.

Many diagnostic microbiology laboratories use the documented observations related to SECs and PMN leukocytes but attempt to coordinate observations related to background materials, which consist of local materials, contaminating materials, and purulence, and to describe the relationship of microorganisms to these background materials. The body site of the sample, such as respiratory samples and wounds, and the classification of the smear together determine the extent of culture evaluation.

Contaminating materials

Contaminating materials (Fig. 7.17) are recognized as materials not coming from the collection site, not contributed by the inflammatory response from the tissues, or not likely to contain the infecting organism. They usually have been added to the specimen in the course of collection from or through a nonsterile area. The most bothersome contaminating

Table 7.7 Murray and Washington's grading system for assessing sputum sample quality

	Epithelial cells per low-power field	Leukocytes per low-power field
Group 1	>25	<10
Group 2	>25	10–25
Group 3	>25	>25
Group 4	10–25	>25
Group 5	<10	>25

Ideally, only samples that fall under groups 4 and 5 are suitable for culture. However, immune-suppressed individuals will have reduced numbers of leukocytes in their secretions as well when their cell counts diminish. Therefore the criteria have been revised so that the number of epithelial cells (>25 per low-power field) is a better indicator of mucosal or saliva contamination.

From Procop, G. W., et al. (2017). *Koneman's color atlas and textbook of diagnostic microbiology* (7th ed.). Philadelphia: Wolters Kluwer.

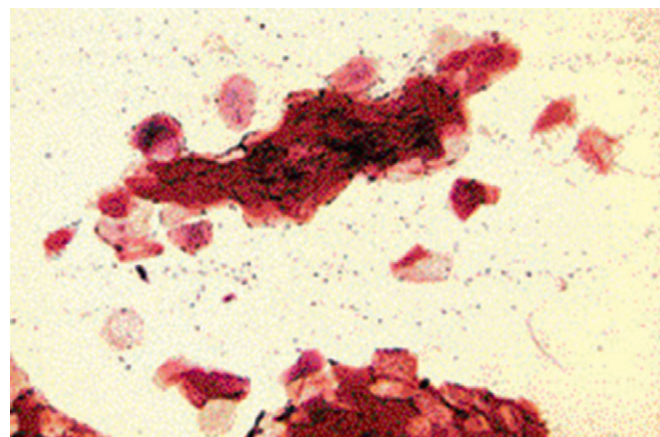


Fig. 7.17 Expecterated sputum smear, Gram stain, light microscopy (low-power view). Purulence, none. Contaminating bacteria and epithelial cells, heavy. No pathogens seen. The sample is saliva, not sputum. There could be several reasons for submission of this sample to the laboratory. The patient could have been poorly directed and simply "spit" into the collection container, or the patient's cough might not be productive of sputum. Another carefully collected sample of lower respiratory tree material should be submitted.

materials are materials containing microorganisms that will grow in culture and potentially confuse the culture interpretation. Expecterated sputum, from the lower lung airways and expelled through the mouth, is the most common contaminated specimen managed by the clinical laboratory.

Criteria

To signify presence of contaminating materials, fewer than 25 PMN leukocytes per low-power field (LPF) and more than 10 epithelial cells or mixed bacteria per LPF must be present.

Direct Gram smear report

The contaminating materials are quantitated as 1+ (light), 2+ (moderate), 3+ (moderately heavy), or 4+ (heavy).

Culture identification guidelines

In a specimen with contaminating material, organism identification should be limited to brief evaluation for *S. aureus*, streptococci or viridans streptococci, *Lactobacillus*, diphtheroids, and β -hemolytic streptococci. Gram-negative rods are reported as enterics (coliforms, non-lactose fermenters, or *Proteus* spp. [spreading colonies]), pseudomonads (oxidase-positive), pathogenic *Neisseria* spp. (identified), *Haemophilus* spp. (smear only), yeasts (note only), and any primary pathogen. When a specimen is regarded as containing contaminating material, a new culture using careful collection technique should be requested.

Antimicrobial susceptibility testing

No antimicrobial susceptibility testing is appropriate except on primary pathogens in specimens with contaminating material.

Local materials

Criteria

Direct smears from specimens containing local materials will likely yield fewer than 25 PMN leukocytes (white blood cells [WBCs]) per LPF and fewer than 10 contaminating epithelial cells per LPF are seen, along with cellular and fluid elements local to the area sampled.

The local constituents may differ as follows:

- Respiratory secretions (Fig. 7.18), including mucus, alveolar pneumocytes (macrophages), ciliated columnar cells, goblet cells, and occasionally metaplastic epithelial cells (smaller than normal SECs)
- CSF (Fig. 7.19) cellular elements
- Cavity fluid (Fig. 7.20) macrophages, a few mixed WBCs, mesothelial cells, and proteinaceous fluid
- Wounds—blood and proteinaceous fluids
- Amniotic fluid (see Fig. 7.16)—anucleate squamous cells and heavy proteinaceous fluid
- Cervix—mucus, columnar epithelial cells, goblet cells, metaplastic SECs, and leukocytes (vary with menstrual cycle)
- Prostatic secretions or semen—spermatozoa and mucus

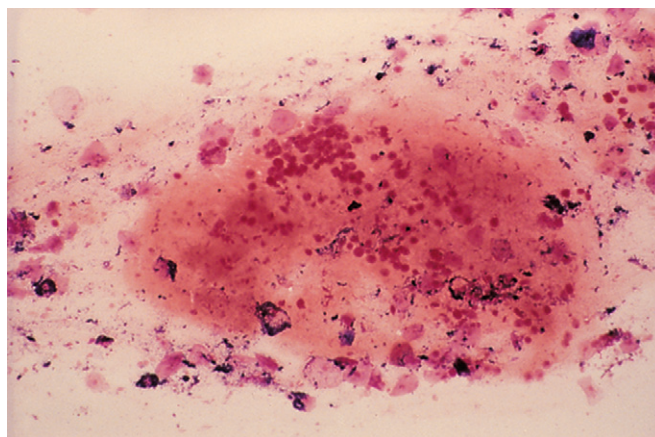


Fig. 7.18 Expectorated sputum, smear, Gram stain, light microscopy (low-power view). Purulence, none. Local materials, moderate. Contaminating bacteria and epithelial cells, heavy. No pathogens seen. The bolus of sputum, consisting of mucus with entrapped alveolar macrophages, confirms that lower respiratory tract material is present. There is no evidence of an infectious process. The sputum is heavily coated by contaminating materials from the oropharynx or mouth. Contaminating organisms will grow in a routine sputum culture.

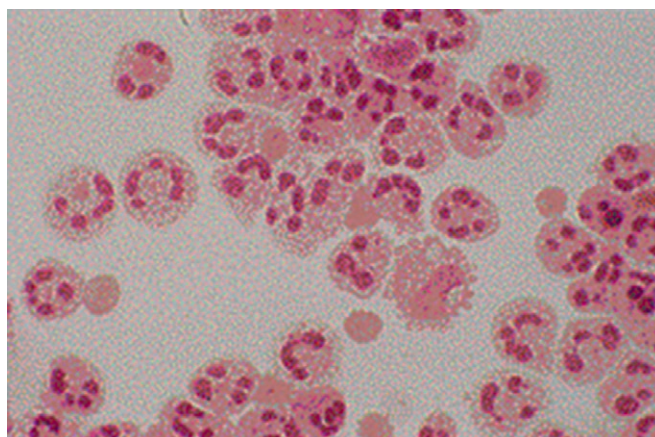


Fig. 7.19 Cerebrospinal fluid (CSF), cytocentrifuge preparation, Gram stain, light microscopy (high-power view). Purulence, moderate. No organisms seen. CSF is a sterile fluid and normally does not have purulence. The presence of neutrophils is critical. Careful observation for bacteria is mandatory. Acridine orange stain may be helpful in clinical settings in which bacteria are low in number and gram-negative. Cytocentrifuged sediments commonly have a concentration of organisms sufficient for routine microscopy ($\geq 10^5$ /mL).

Direct Gram smear report

Local microbial biota is quantitated as 1+ to 4+ (see the “Contaminating Materials” section earlier in this chapter).

Culture identification guidelines

The designation “usual microbiota” or a brief presumptive description of “colony-type growth” may be used (see the “Contaminating Materials” section earlier in this chapter).

Antimicrobial susceptibility testing

No antimicrobial susceptibility testing is appropriate on specimens with local material.

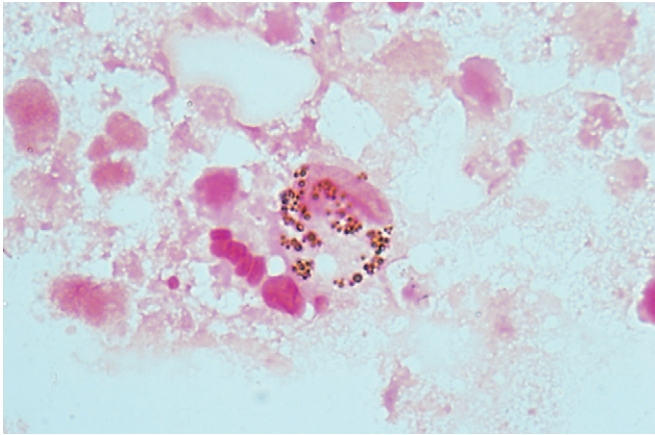


Fig. 7.20 Eye, vitreous aspirate, smear, Gram stain, light microscopy (high-power view). Purulence, light. Amorphous debris, moderate. Local materials, light. No organisms seen. This smear suggests that there has been an injury. The pigment has undergone phagocytosis and is seen within a large macrophage. Pigment must not be mistaken for bacteria, and bacteria must never be overlooked if mixed with local materials, such as pigment granules.

Purulence

Criteria

Greater than 25 PMN leukocytes (WBCs) per LPF and fewer than 10 epithelial cells per LPF are seen. Mucus or heavy proteinaceous material may be present. The clinical material can have a foul odor.

Direct Gram smear report

Only organisms intimately associated with the WBCs, mucus, or proteinaceous exudate are quantitated. The following system is used: 1+ (rare organisms per oil immersion field [OIF]), 2+ (few organisms per OIF), 3+ (moderate number per OIF), and 4+ (many per OIF). Contaminating materials are quantitated separately—should be 1+ (none or few).

Culture identification guidelines

Colony growth should be correlated with Gram-stained smear, as in the Case in Point. *S. pneumoniae* from viridans streptococci, β -hemolytic streptococci (Lancefield groups A, B, and D; C, F, and G, if indicated clinically), *S. aureus*, *H. influenzae*, pathogenic *Neisseria* spp., gram-negative bacilli, yeasts (*C. neoformans* only; note the presence of other genera), filamentous fungi (transparent tape preparation in biological safety cabinet), and other organisms are identified as indicated by smear findings.

Antimicrobial susceptibility testing

S. aureus, gram-negative nonfastidious bacilli, and other organisms should be tested for as appropriate or specifically requested. Studies have demonstrated that patients with microscopic evidence of purulence with positive cultures are more likely to receive antimicrobial therapy. However, microscopic evidence of purulence is not always associated with infection.

Mixed materials

Mixed materials consist of purulent exudate, contaminating materials, and local materials in a single smear.

Criteria

Fewer than 25 PMN leukocytes (WBCs) per LPF and fewer than 10 epithelial cells or contaminating bacteria per LPF are seen. Local secretions may also be present.

Direct Gram smear report

For mixed materials, only organisms intimately associated with purulent exudate are quantitated. The amounts of other elements, contaminating materials, and local materials also are quantitated.

Culture identification guidelines

A new culture may be requested for mixed specimens. A new specimen must be requested if the specimen shows the presence of purulence and the culture results cannot be interpreted. If a new culture specimen cannot be obtained, evaluation should proceed as for contaminating materials.

Antimicrobial susceptibility testing

Purulence guidelines should be used for testing organisms that appear significant.

Reports of direct examinations

Reports of the results of direct specimen examination should be made available as soon as they are completed. The availability of computer-managed reporting (i.e., hospital information systems) using direct physician access through computer terminals in patient care areas facilitates immediate reporting of results. Reports of direct examinations should be simple and include all information elements needed by the physician to understand the report. The report lists the type of material submitted and clearly states whether the observed microbes are of significance. An example of computer-managed microscopic reports from direct specimen examinations such as at The Ohio State University is shown in the samples given in [Box 7.1](#).

Only elements useful in characterizing the specimen should be included in the report. Interpretative comments also may be included when, on the basis of specimen type, background materials, and organism morphology, there is little doubt about the nature of the process or the offending infectious agent. All telephone reports of direct examinations should be recorded in the report. Once this stepwise evaluation of the smear has been completed, the information yielded, along with the clinical setting, allows reasonable management of infected patients until the subsequent steps of culture isolation, susceptibility testing, or antibody or antigen detection can occur.

BOX 7.1 Sample reports of direct microscopic examinations

Respiratory culture	Acc. no. XXXX
Source:	Sputum: expectorated
Microscopic	Purulence heavy Contaminating bacteria, yeasts, and epithelial cells heavy Gram-positive diplococci: consistent with pneumococci

Called to Dr. Doe at 8:00 PM 4/13/21

Respiratory culture	Acc. no. XXXXX
Source:	Sinus: ethmoid contents
Microscopic	Purulence moderate Local materials light Red blood cells present Gram-negative bacilli: consistent with <i>Pseudomonas</i>

Called to Dr. Doe at 11:35 AM 4/5/21

Case check 7.2

The foundation in the diagnostic microbiology laboratory, as in the Case in Point, is the ability to combine the rapid response of direct specimen examination with culture isolation and antimicrobial susceptibility testing to achieve the following:

- Confirm that the material submitted for study is representative.
- Identify the cellular components and debris of inflammation and establish the probability of infection.
- Identify specific infectious agents using direct visual detection of characteristic shape, size, and Gram stain reaction.
- Augment visual identification of microbes by use of specific stains, or antibody or gene-directed probes.
- Provide antimicrobial susceptibilities of isolated pathogens to guide treatment.
- Develop epidemiologic data.



Fig. 7.21 Sheep blood agar plate inoculated with expectorated sputum, 24-hour incubation with 5% carbon dioxide in air. Note heavy bacterial growth in the area of primary inoculation with thin trails of colonies (arrows) lacing the surface of the agar (see Fig. 7.22).

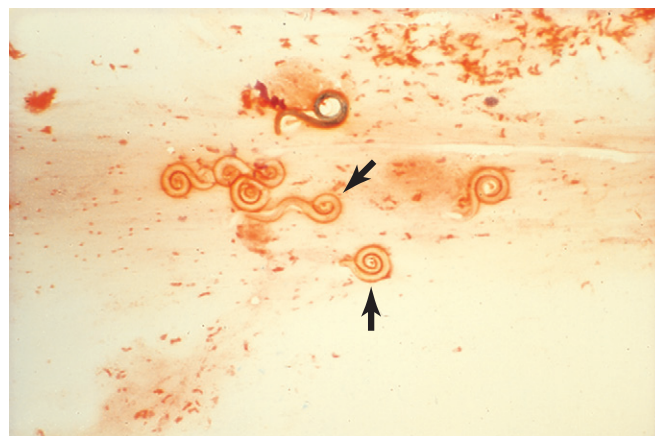


Fig. 7.22 Aspirated sputum, Gram stain, light microscopy (low-power view). Purulence, none. Local materials, moderate. Mucus present. Coiled nematode larvae (arrows). Morphology consistent with *Strongyloides stercoralis*. Impression: *Strongyloides* hyperinfestation syndrome.

Initiation of special handling for unsuspected or special pathogens

The direct microscopic examination of infected materials, along with specimen site and historic information, may suggest modifications in routine culture techniques to allow isolation of a suspected pathogen. Modifications might involve ordering other culture tests, adding special media, increasing incubation time, or changing incubation temperature or atmosphere. If recognition or suspicion of such pathogens does not occur from smear or history, the isolation of certain pathogens will not be made, and diagnosis will be delayed or missed.

Some pathogens do not grow in culture and are usually unsuspected. One is the nematode parasite *Strongyloides stercoralis*, which can be seen in the sputum and bronchoalveolar fluid of patients with hyperinfestation syndrome (Figs. 7.21 and 7.22). Visual recognition can lead to a request for stool examination for parasite load and prompt treatment. Untreated hyperinfestations are associated with death.

Pathogens that require special culture media for laboratory isolation can be recognized on smear and redirected for appropriate culture. Organisms such as *Legionella* spp., *Mycobacterium* spp., and *Bartonella* spp. must be placed on appropriate media for culture confirmation of infection.

Quality control in direct microscopic interpretations

QC considerations such as the quality of specimens submitted, how well the staining procedure was performed, and the adequacy of culture interpretation can be monitored using the results of direct examination. The quality of the staining reagents and proper staining technique can be assessed using organisms such as *S. aureus* and *Escherichia coli* to serve as the positive and negative controls, respectively. QC practice that monitors both the smear and the culture interpretation should be an ongoing work activity. Correlation between the two results should be made for each patient. Explanations for discrepant results should be

sought within the work material. This repeated inspection of results enables each observer to practice self-education and improve observation skills. Review of these QC activities allows corrections to be made in specimen collection, specimen management, materials and procedure administration, and culture management.

Quality improvement activities often are suggested by the results of QC monitoring of direct specimen examination. For example, it may be documented that sputum specimens of poor quality are consistently submitted from certain physicians' offices, clinics, or nursing stations in the hospital. This observation becomes the basis for planning corrections that move outside the laboratory to involve the population of patients served.

Examples of sample observations and reports

Additional examples of direct observations and the associated reports and comments are given in [Plates 1 to 61](#). Practice to determine whether you can make a similar report using only the observation provided. Then read each report to see whether you obtain similar impressions of the specimen and pathogen from the observation and written report.

POINTS TO REMEMBER

Direct microscopy of materials submitted to the laboratory for identification of infectious organisms offers the first, last, and best opportunity to do the following:

- Suspect probable infection.
- Confirm the diagnostic quality of the specimen submitted.
- Recognize "classic" organism morphotypes associated with infection.
- Recognize pathogens that will not grow in culture or the culture type requested, thus requiring a different diagnostic approach.
- Provide a "stat" laboratory response to serious infection.
- Provide a pathway to confirm the suspected cause of infection

LEARNING ASSESSMENT QUESTIONS

- c. Calcofluor white stain
- d. All of the above
4. Which of the following stains is best used to detect mycobacterial organisms in clinical samples?
 - a. Gram
 - b. Giemsa
 - c. Acid-fast
 - d. India ink
5. Calcofluor white is a colorless dye that binds with which of the following structures?
 - a. Chitin
 - b. Mycolic acid
 - c. Peptidoglycan layer
 - d. Metachromatic granules
6. Cyto centrifugation is an excellent method for which of the following types of samples?
 - a. Heavily contaminated
 - b. Nonviscous fluids
 - c. Thick purulent material
 - d. Bloody specimens
7. Which of the following is indicative of a monobacterial type of infection based on the direct microscopic examination of the clinical sample?
 - a. Keratohyalin granules
 - b. Inflammatory debris
 - c. Squamous epithelial cells
 - d. A single morphotype of microorganism
8. In a Wright-Giemsa-stained smear, bacteria would appear as which of the following colors?
 - a. Red
 - b. Green
 - c. Blue
 - d. Orange
9. In preparing smears from swabs, which of the following should be followed?
 - a. Inoculate the solid culture media first with the swab before preparing the smear on the slide.
 - b. Roll the swab back and forth over the selected area on the slide.
 - c. Rub the swab back and forth spreading the material across the desired area on the slide.
 - d. Place the swab in a broth medium before applying the material on the slide.
10. Which of the following is the value of a direct microscopic examination for microorganisms in clinical samples?
 - a. Results may confirm or reject the physician's initial diagnosis.
 - b. Useful results keep the treatment plan moving in the right direction when delivered in a timely manner.
 - c. Direct smear examination results can be correlated with culture results.
 - d. All of the above.
11. When examining the Gram stain quality control slides, you note that *S. aureus* and *E. coli* stained pink. Which of the following is likely?
 - a. The results are correct.
 - b. The two smears are under-decolorized.
 - c. The *S. aureus* smear is over-decolorized.
 - d. The *E. coli* smear is over-decolorized.

1. Direct smear examination of clinical samples is a rapid means to identify presumptively the etiologic agents of infectious disease. True or false?
2. The presence of an infectious disease process can be assessed on a direct smear based on which of the following?
 - a. The presence of numerous inflammatory cells
 - b. The morphology of the bacteria present
 - c. Gram stain reaction of bacteria present
 - d. The presence of numerous epithelial cells
3. Which of the following stains may be used for direct smear examination?
 - a. Gram stain
 - b. Acid-fast stain

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Direct examination showing local and contaminating materials

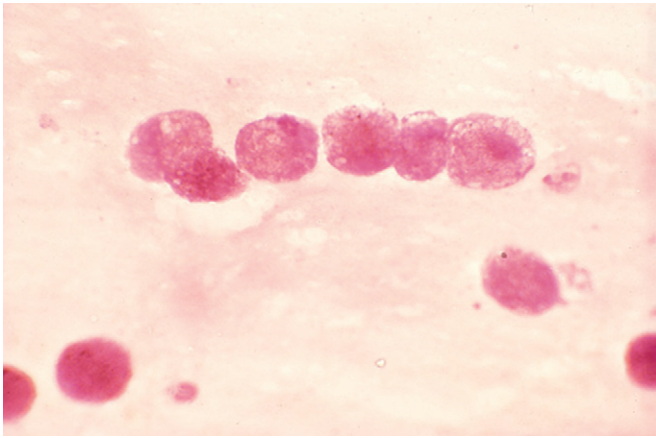


PLATE 1 Aspirated sputum, smear, Gram stain, light microscopy (high-power view). Purulence, none. Local materials, moderate. No organism seen. The alveolar macrophages and mucus (pink-stained background) are the local materials from the tracheobronchial tree. This smear confirms that sputum was sampled, and there is no suspicion of infection and no evidence of significant contamination. Routine culture of this specimen can grow insignificant oral biota because culture is more sensitive than direct examination.

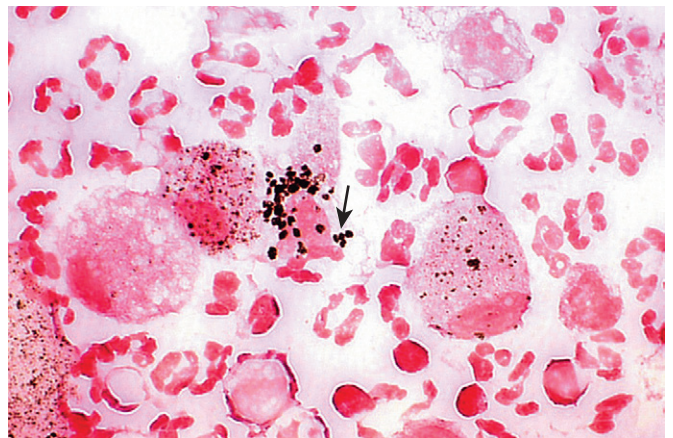


PLATE 3 Bronchoalveolar lavage, cytocentrifuge smear, Gram stain, light microscopy (high-power view). Purulence, heavy. No organisms seen. Local materials, black particulate debris (*arrow*). This size and type of carbon particles is commonly seen in respiratory samples in small amounts and is usually not noted in a smear report.

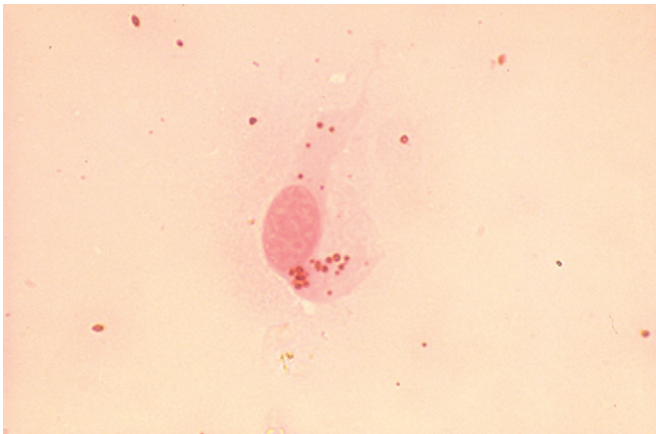


PLATE 2 Trauma eye, vitreous aspirate, smear, Gram stain, light microscopy (high-power view). Purulence, none. No organisms seen. Local materials, light. The light protein background and the pigment-containing cell are normal material local to the vitreous of the eye. This local material confirms that the sample is representative. The ability to see this brown pigment cell in smear material is related to the eye trauma. The important emphasis is the absence of purulence.

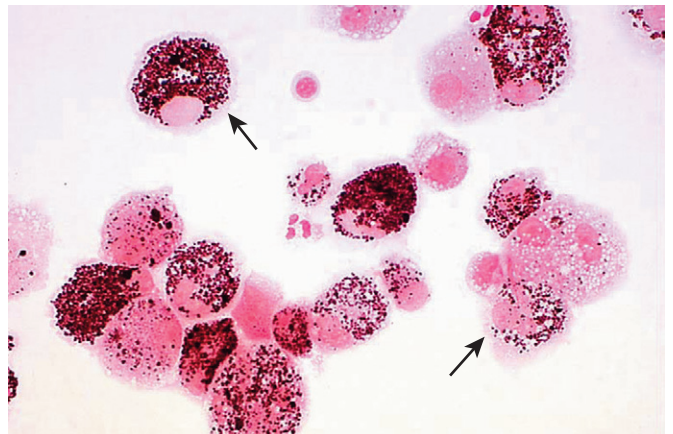


PLATE 4 Bronchoalveolar lavage, cytocentrifuge smear, Gram stain, light microscopy (high-power view). Purulence, none. No organisms seen. Local materials, heavy black particulate debris (*arrows*). This small carbon particles seen prominently in respiratory samples can be associated with smoking crack cocaine. The gross sample usually has a gray appearance. Black particulate debris should not be confused with gram-positive cocci.

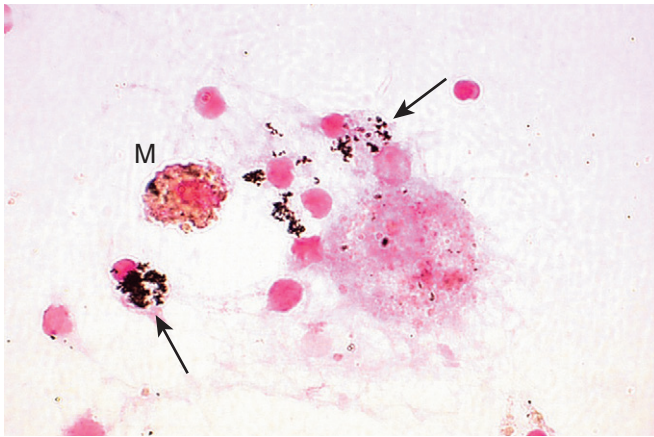


PLATE 5 Bronchoalveolar lavage, cytocentrifuge smear, Gram stain, light microscopy (high-power view). Purulence, none. No organisms seen. Local materials, black particulate debris (BPD) moderate. This BPD is irregular in shape and usually varies in size (*arrows*). This type of carbon debris is commonly associated with smoke inhalation from house or other types of fires. In the early phase of smoke inhalation, much of the BPD is extracellular. Later, much of the debris is intracellular within phagocytes. The golden macrophage present contains yellow material associated with cigarette smoking.

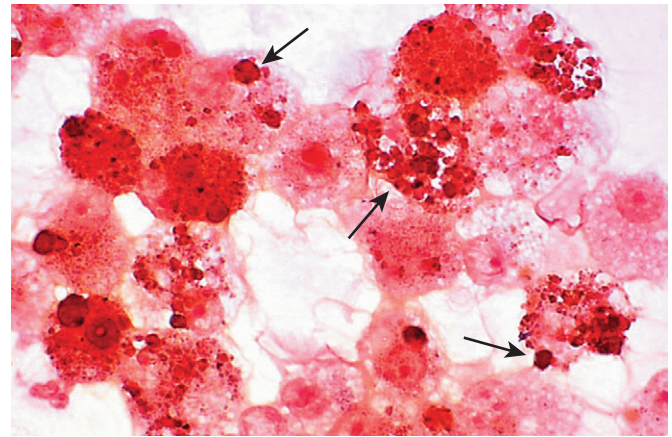


PLATE 7 Bronchoalveolar lavage, cytocentrifuge smear, Gram stain, light microscopy (high-power view). Purulence, none. No organisms seen. Local materials, alveolar macrophages containing golden yellow to Gram's safranin-colored chunky, irregular particles consistent with hemosiderin (*arrows*). This type of large-particle hemosiderin deposition within lung phagocytes is commonly associated with blood in the lung as seen in heart failure or aspirated blood from large-volume nosebleeds. Blood and hemosiderin in a Gram-stained smear make smear viewing more difficult.

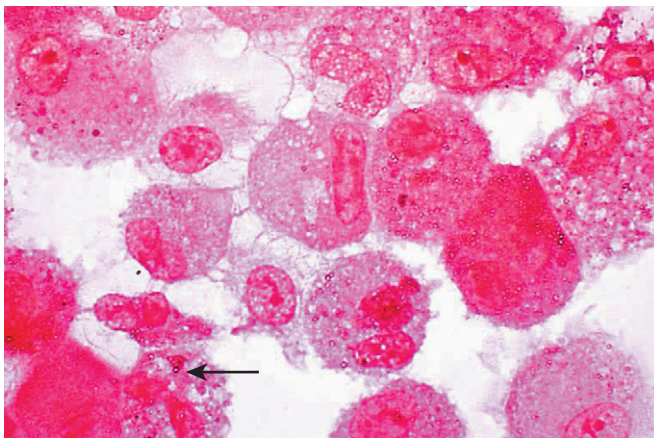


PLATE 6 Bronchoalveolar lavage, cytocentrifuge smear, Gram stain, light microscopy (high-power view). Purulence, none. No organisms seen. Local materials, alveolar macrophages containing very small, light to golden yellow, refractile but not polarizing particles (*arrow*). These small, fine particles can be hemosiderin, sometimes deposited as small particles or other fine particles from the environment. Such refractile particles are not usually reported except in response to specific questions from the patient's physician.

Direct examination in common bacterial infections

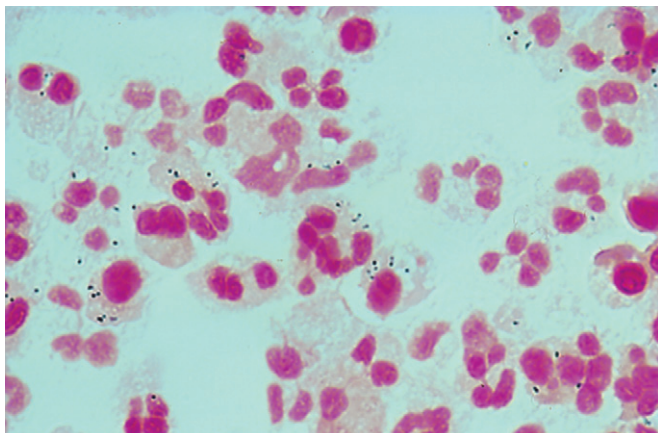


PLATE 8 Expectorated sputum, smear, Gram stain, light microscopy (high-power view). Purulence, heavy. The presence of gram-positive diplococci, intracellular morphology suggests an antimicrobial agent effect. *Impression:* pneumococcal disease. This is a typical smear presentation of a treated but unresolved pneumococcal pneumonia. Neutrophils cover the field, the diplococci are largely intracellular and partially digested, and the background amorphous material is gone. Routine bacterial culture of this sample may be negative for typical colonies of *S. pneumoniae*. A few colonies may be found by a careful search among the contaminating normal biota colonies.

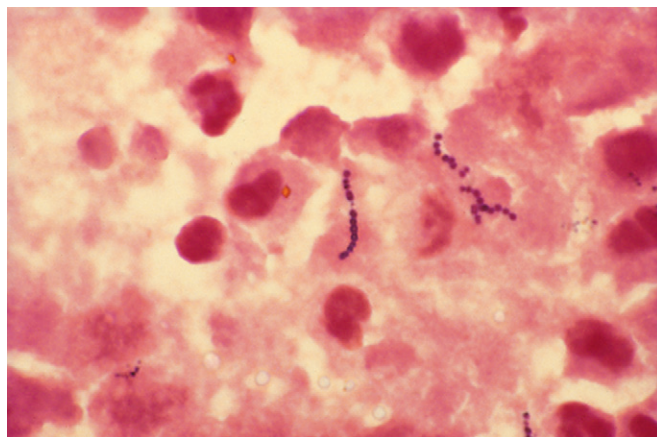


PLATE 10 Wound, smear, Gram stain, light microscopy (medium-power view). Purulence, moderate. Amorphous debris, moderate. Gram-positive cocci, chains, extracellular. *Impression:* streptococcal disease. The presence of typical chains of *Streptococcus* on a background showing purulence with poorly preserved polymorphonuclear (PMN) leukocytes and amorphous debris is suggestive of hemolytic streptococci with tissue cytotoxicity. Routine bacterial culture yielded a pure growth of *S. pyogenes*.

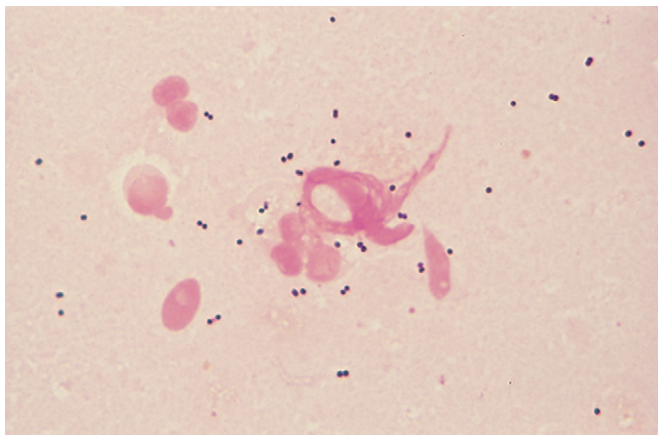


PLATE 9 Aspirated sputum, smear, Gram stain, light microscopy (medium-power view). Purulence, light. Amorphous debris, heavy. Gram-positive cocci, pairs, encapsulated, extracellular. Initial antimicrobial therapy can be directed toward streptococci and staphylococci (*Stomatococcus*). Routine bacterial culture isolated a pure growth of an encapsulated strain of *Streptococcus pyogenes*. The heavy amorphous background is protein-rich edema fluid from the capillary bed damaged by *S. pyogenes* toxins. The patient subsequently died of the infection despite a correct diagnosis and appropriate antimicrobial therapy.

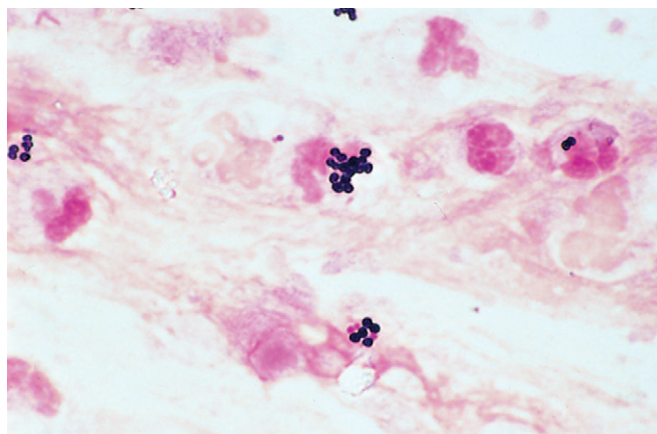


PLATE 11 Expectorated sputum, smear, Gram stain, light microscopy (medium-power view). Purulence, light. Local materials, moderate. Gram-positive cocci, pairs, groups, intracellular, and extracellular. *Impression:* staphylococcal disease. Smear morphology is typical for staphylococci, but no staphylococcal colonies were present on the culture plates. *Stomatococcus mucilaginosus* colonies were present in high numbers. Careful correlation between direct and culture examinations demonstrated this organism to be the probable cause of infection. The presumptive report implying or suggesting staphylococci followed by a negative culture report without explanation raises doubts about the competence of the laboratory.

Direct examination in gram-positive bacillary infections

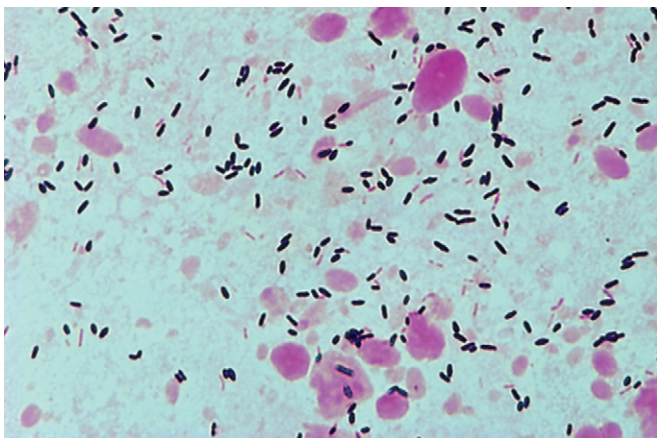


PLATE 12 Amniotic fluid, cytocentrifuge, Gram stain, light microscopy (medium-power view). Purulence, light. Local materials, moderate. Gram-positive bacilli, small. Morphology consistent with *Listeria monocytogenes*. Impression: congenital listeriosis.

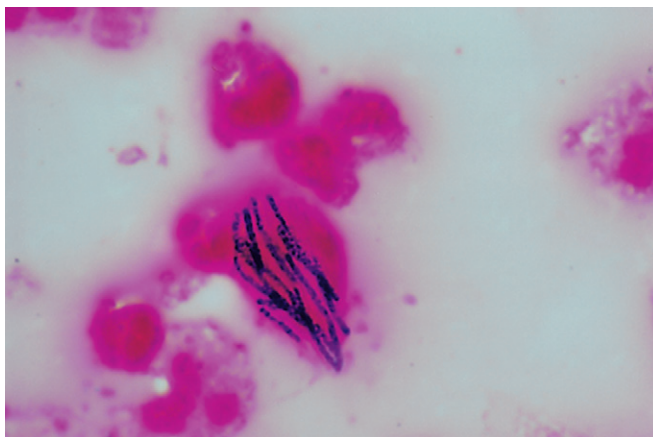


PLATE 15 Amniotic fluid, cytocentrifuge preparation. Gram stain, light microscopy (high-power view). Purulence, light. Local materials, moderate. Gram-positive bacilli, medium, long, intracellular. Morphotype consistent with *Lactobacillus* spp. Impression: amnionitis.

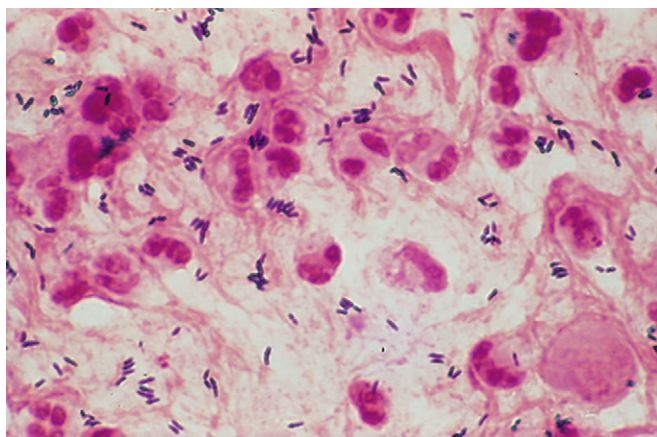


PLATE 13 Expectorated sputum, smear, Gram stain, light microscopy (medium-power view). Purulence, moderate. Local materials, moderate. Gram-positive bacilli, diphtheroid. Morphology suggests coryneform infection. Routine bacterial culture grew *Corynebacterium pseudodiphtheriticum*.

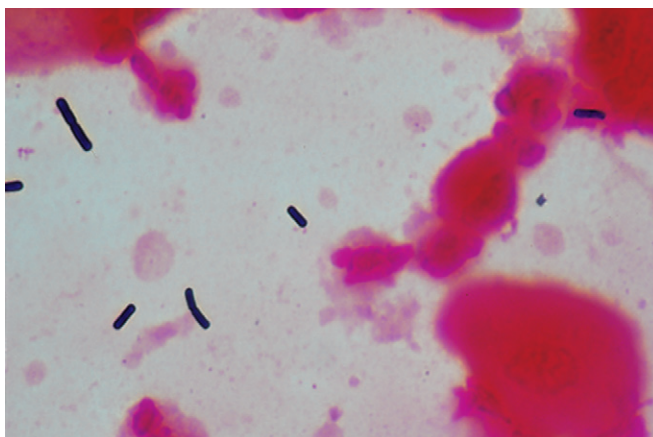


PLATE 16 Amniotic fluid, smear, Gram stain, light microscopy (medium-power view). Purulence, light. Local materials, moderate. Gram-positive bacilli, large. Morphology consistent with *Clostridium perfringens*. Impression: amnionitis.

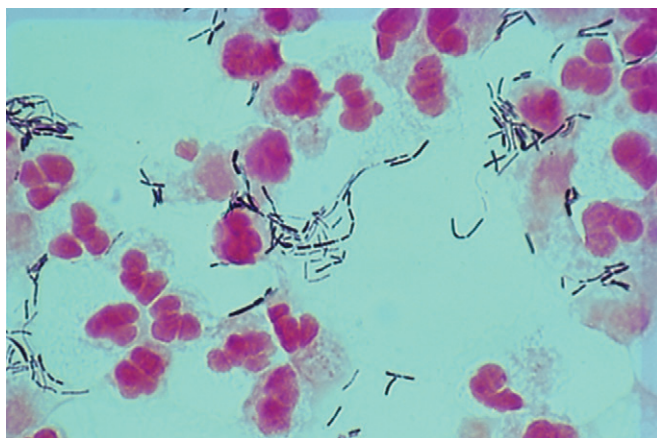


PLATE 14 Urine, cytocentrifugation, Gram stain, light microscopy (medium-power view). Purulence, moderate. Gram-positive bacilli, medium, long, chaining. Morphology consistent with *Lactobacillus* spp. Impression: cystitis.

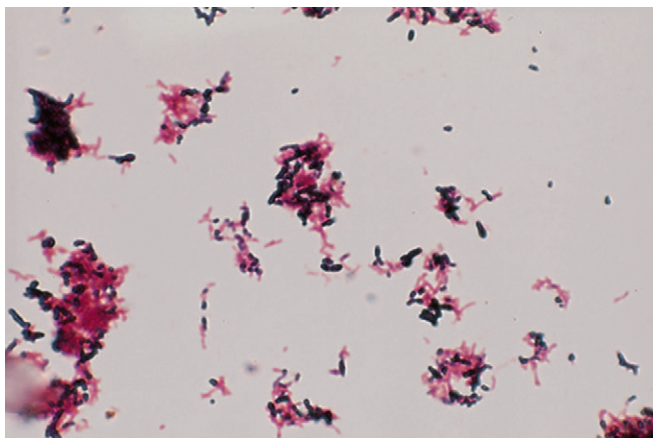


PLATE 17 Vaginal culture, colony from blood agar, smear, Gram stain, light microscopy (high-power view). Gram-positive bacilli, diphtheroid, variably Gram staining. Morphotype consistent with *Gardnerella vaginalis* (see Plate 18).

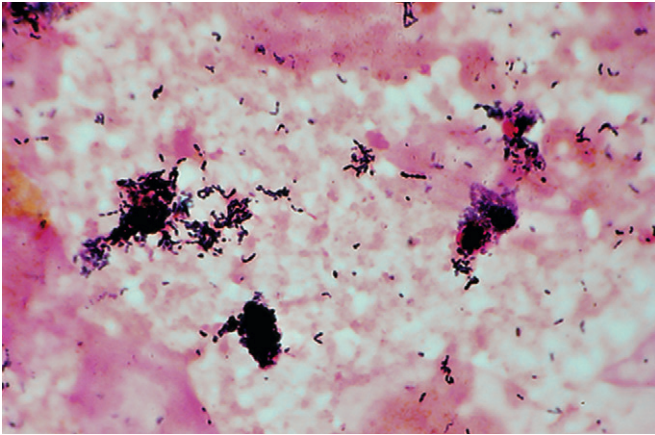


PLATE 18 Amniotic fluid, smear, Gram stain, light microscopy (high-power view). Local materials, heavy. Gram-positive bacilli, diphtheroid, variably Gram staining. Morphotype consistent with *Gardnerella vaginalis*. Impression: amnionitis.

Direct examination in uncommon gram-positive bacilli

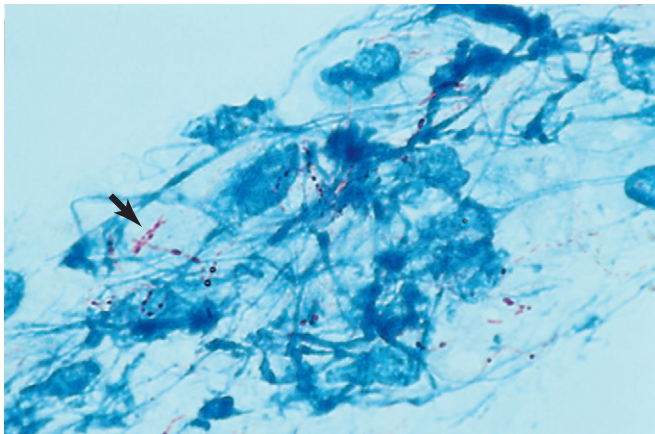


PLATE 19 Abscess aspirate, smear, acid-fast stain (Ziehl-Neelsen), light microscopy (medium-power view). Purulence, moderate. Acid-fast bacilli, numerous (arrow). Mycobacterial cultures grew *Mycobacterium kansasii*.

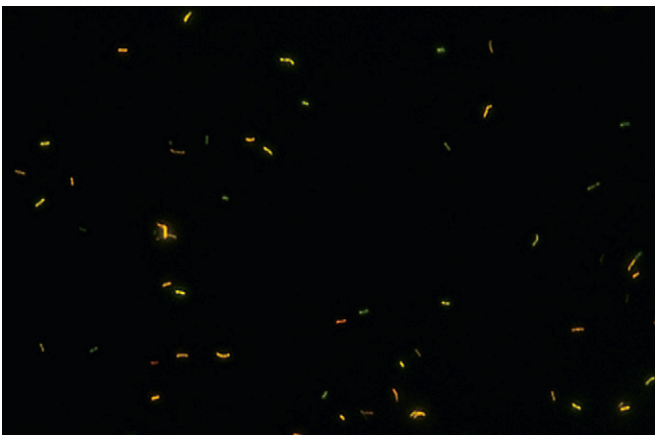


PLATE 20 Expecterated sputum, concentrated smear, fluorochrome acid-fast stain, fluorescent microscopy (medium-power view). Typical acid-fast bacteria, numerous. Impression: mycobacterial disease. The patient had been placed in respiratory isolation after physical examination and history taking, and antituberculosis therapy was immediately begun after receipt of the direct examination report. *Mycobacterium tuberculosis* was identified from culture.

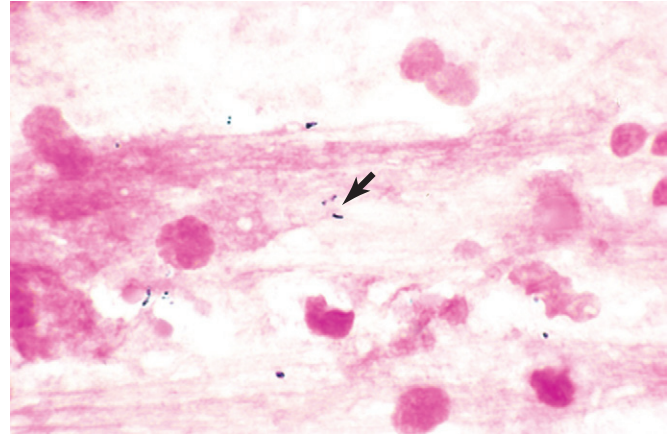


PLATE 21 Expecterated sputum, smear, Gram stain, light microscopy (medium-power view). Purulence, light. Local materials, light. Amorphous debris, moderate. Gram-positive bacilli, medium, beaded (arrow). Follow-up acid-fast stain positive. Chest radiograph with right lung upper lobe mass. Culture yielded *Rhodococcus equi*.

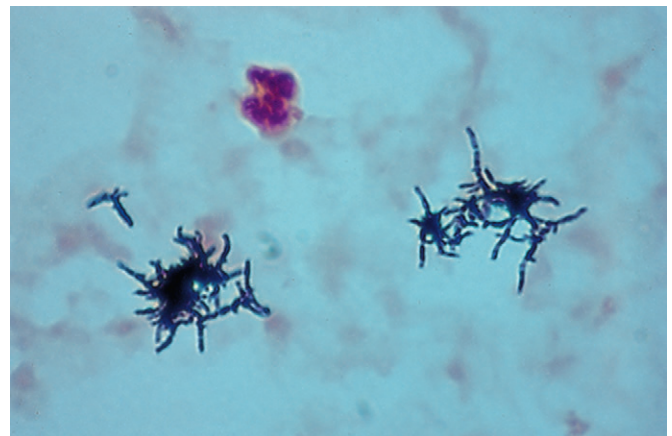


PLATE 22 Blood culture, sediment smear, Gram stain, light microscopy (medium-power view). Blood. Gram-positive bacilli, branched, beaded. Morphology consistent with *Actinomyces* or *Propionibacterium* spp. Culture isolation of *Actinomyces israelii*.



PLATE 23 Expectorated sputum, smear, Gram stain, light microscopy (medium-power view). Purulence, light. Local materials, light. Amorphous debris, moderate. Gram-positive bacilli, branched, beaded. Morphotype consistent with *Nocardia* or *Actinomyces*.

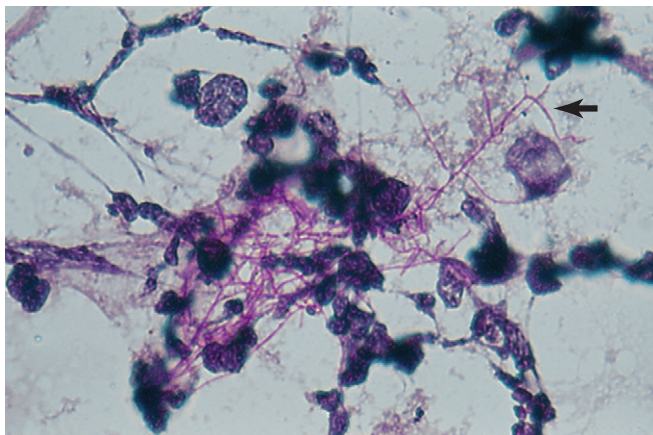


PLATE 24 Expectorated sputum, smear, partial acid-fast stain, light microscopy (medium-power view). Partially acid-fast bacilli, branched, beaded (arrow). Morphology consistent with *Nocardia* spp. *Impression:* nocardiosis.

Direct examination in gram-positive bacilli with filaments and branches

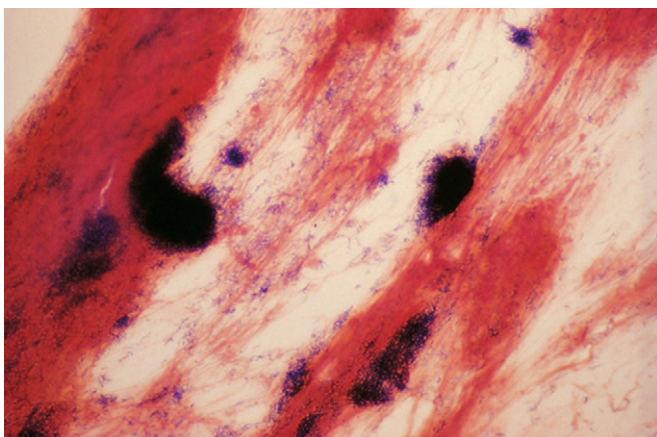


PLATE 25 Jaw abscess aspirate, smear, Gram stain, light microscopy (low-power view). Purulence, heavy. Granules present. Suspicious for *Actinomyces* spp. (see Plate 27).

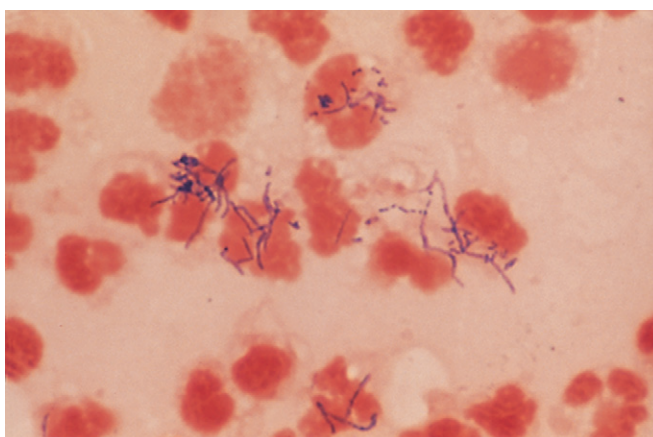


PLATE 27 Cutaneous sinus tract aspirate, smear, Gram stain, light microscopy (high-power view). Purulence, heavy. Gram-positive bacilli, filamentous, beaded, branched, partial acid-fast stain-negative. Morphology consistent with *Actinomyces* spp. *Impression:* actinomycosis.

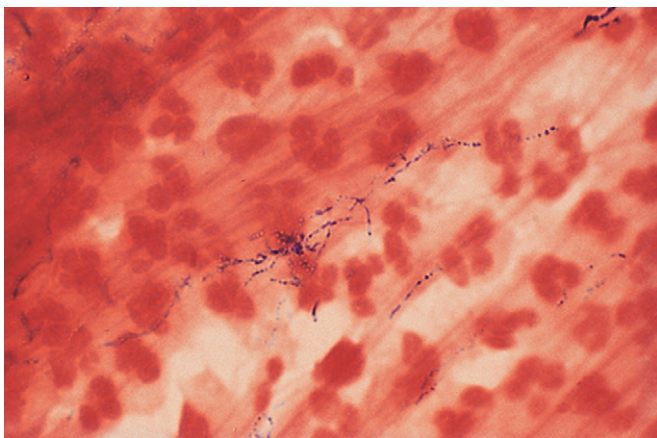


PLATE 26 Jaw abscess aspirate, smear, Gram stain, light microscopy (high-power view). Purulence, heavy. Gram-positive bacilli, filamentous, beaded, branched, partial acid-fast stain negative. Morphology consistent with *Actinomyces* spp. *Impression:* actinomycosis (lumpy jaw).

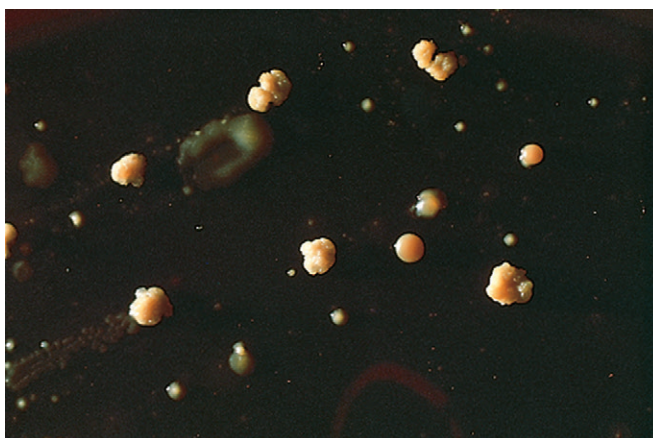


PLATE 28 Cutaneous sinus tract aspirate, colonies on an anaerobic blood agar plate. Mixed colony morphotypes. Molar tooth colonies. Morphology consistent with *Actinomyces israelii*. *Impression:* mixed anaerobic infection—actinomycosis.

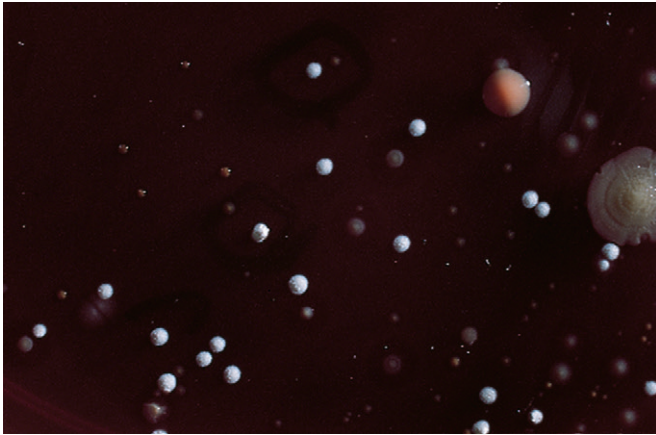


PLATE 29 Expecterated sputum, chalky white colonies on plate with 5% sheep blood agar at 5 days' incubation. Routine sputum culture. Morphology consistent with *Nocardia*, *Nocardiosis*, or *Streptomyces* spp.

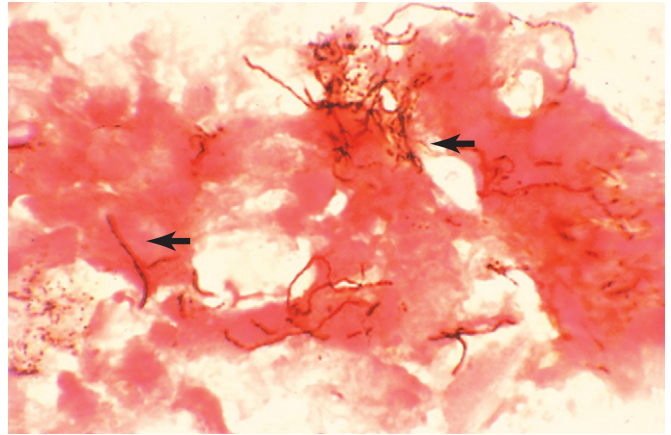


PLATE 31 Surgical biopsy of abnormal area in jawbone, smear, Gram stain, light microscopy (high-power view). Amorphous debris, heavy. Gram-positive bacilli, filamentous, beaded, branched, partial acid-fast stain-negative (*arrows*). Morphology suggests actinomycete. Aerobic and anaerobic cultures negative.

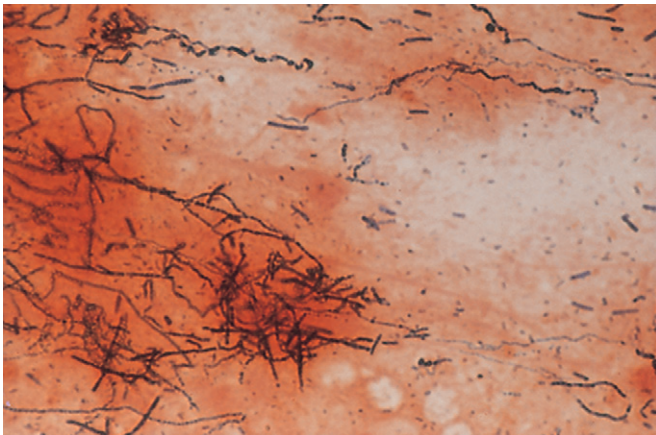


PLATE 30 Sinus tract granule, crush-pull smear, Gram stain, light microscopy (high-power view). Amorphous debris, moderate. Gram-positive bacilli, filamentous, beaded, branched, partial acid-fast stain-negative. Gram-positive bacilli, regular. Gram-negative bacilli, small. Gram-positive cocci. Morphology suggests mixed anaerobic infection with actinomycetes. *Impression*: actinomycosis. Culture isolation of *Actinomyces naeslundii*.

Direct examination in selected gram-negative bacterial infections

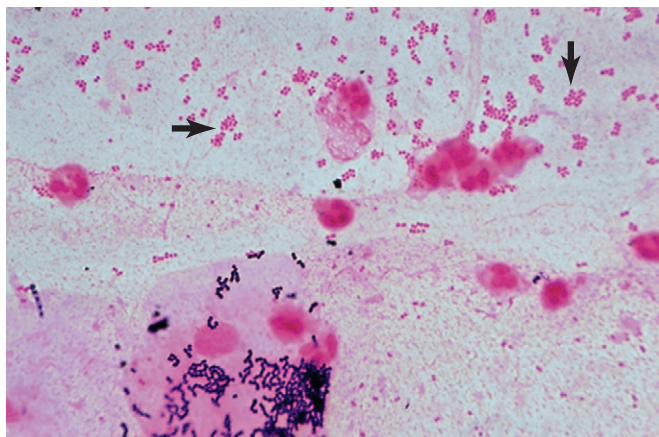


PLATE 32 Expectorated sputum, smear, Gram stain, light microscopy (medium-power view). Mixed materials, type I, layered. Contaminating bacteria and epithelial cells, moderate. Purulence, light. Gram-negative diplococci (arrows). Morphology suggests pathogenic *Neisseria* or *Moraxella* spp. Routine bacterial culture isolated *Moraxella catarrhalis*.

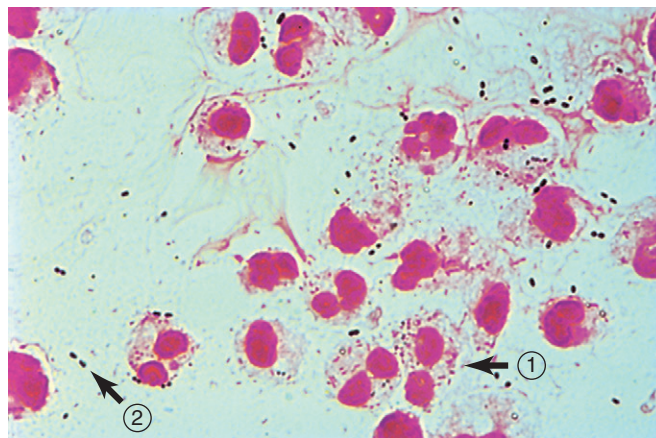


PLATE 34 Expectorated sputum, smear, Gram stain, light microscopy (high-power view). Purulence, heavy. Local materials, moderate. Gram-negative bacilli, small, pleomorphic, intracellular, extracellular (arrow 1). Gram-positive diplococci, encapsulated, intracellular, extracellular (arrow 2). Morphology consistent with *H. influenzae* and *S. pneumoniae*. Impression: polymicrobial infection.

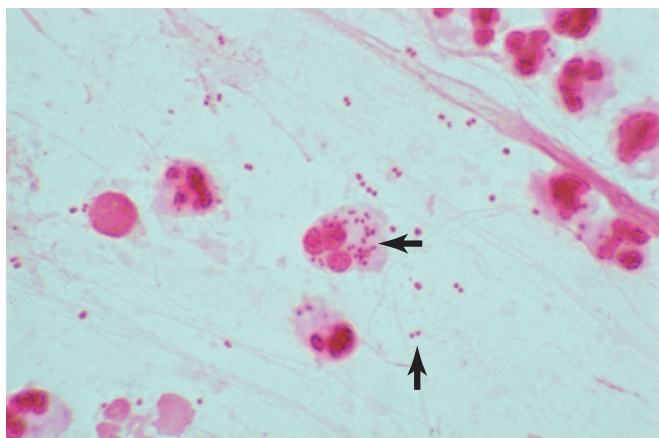


PLATE 33 Expectorated sputum, smear, Gram stain, light microscopy (medium-power view). Purulence, moderate. Local materials, moderate. Gram-negative diplococci, intracellular, extracellular (arrows). Morphology suggests pathogenic *Neisseria* or *Moraxella* spp. Routine bacterial culture isolated *Neisseria meningitidis*.

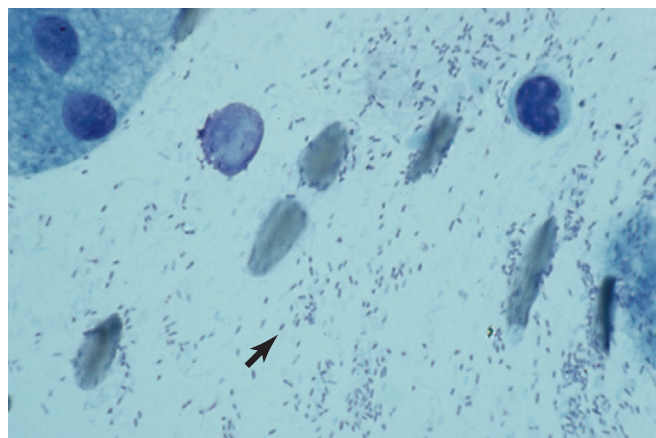


PLATE 35 Bronchoalveolar lavage, cytocentrifuge preparation, Wright-Giemsa stain, light microscopy (high-power view). Purulence, none. Lymphocytes present. Local materials, moderate. Small bacilli (arrow), numerous (see Plate 36).

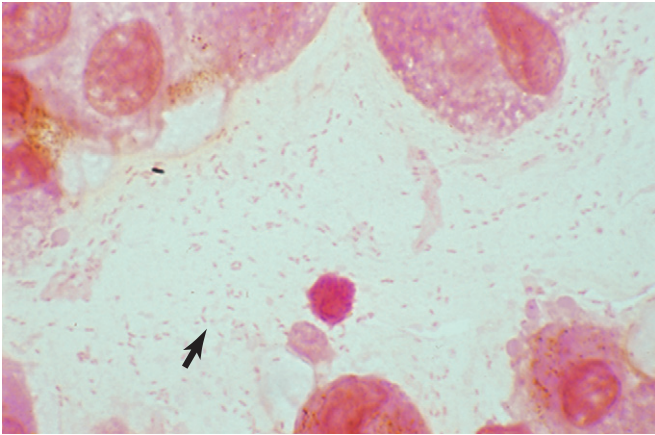


PLATE 36 Bronchoalveolar lavage, cytocentrifuge preparation, Gram stain, light microscopy (high-power view). Purulence, none. Lymphocytes present. Local materials, moderate. Gram-negative bacilli (arrow), small (see Plate 37).

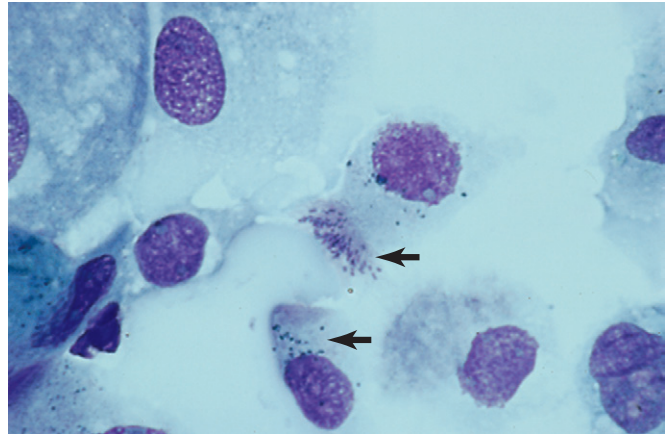


PLATE 38 Bronchoalveolar lavage, cytocentrifuge preparation, Wright-Giemsa stain, light microscopy (high-power view). Purulence, none. Lymphocytes present. Local materials, moderate. Ciliated columnar epithelial cells with numerous small bacilli adherent to cilia (arrows). Morphology consistent with *Bordetella pertussis*. Impression: whooping cough (pertussis).

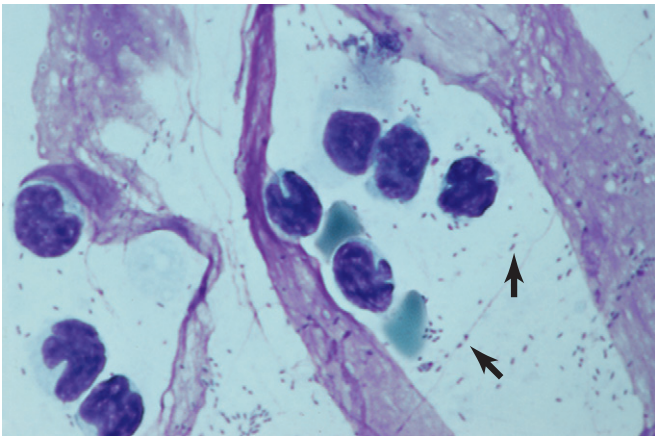


PLATE 37 Bronchoalveolar lavage, cytocentrifuge preparation, Wright-Giemsa stain, light microscopy (high-power view). Purulence, none. Lymphocytes present. Local materials, moderate. Bacilli (arrows), small.

Direct examination in selected gram-negative bacillary infections

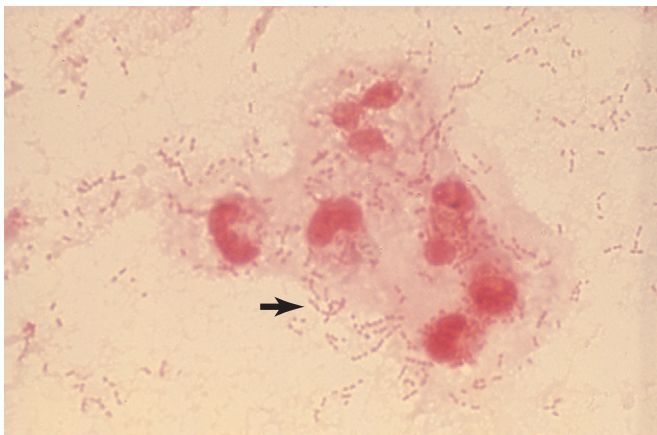


PLATE 39 Cerebrospinal fluid, drop smear, Gram stain, light microscopy (high-power view). Purulence, moderate. Gram-negative coccobacilli, chains (arrow). Morphotype suggests *Bacteroides* spp. *Impression:* Gram-negative bacillary meningitis.

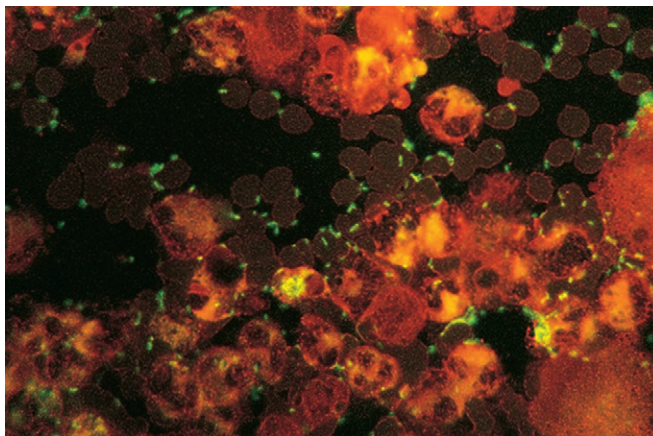


PLATE 42 Bronchoalveolar lavage, cytocentrifuge preparation, direct fluorescent antibody. *Legionella pneumophila*, polyvalent antisera, fluorescent microscopy (high-power view). Immunofluorescence-positive. *Impression:* Legionnaires' disease.

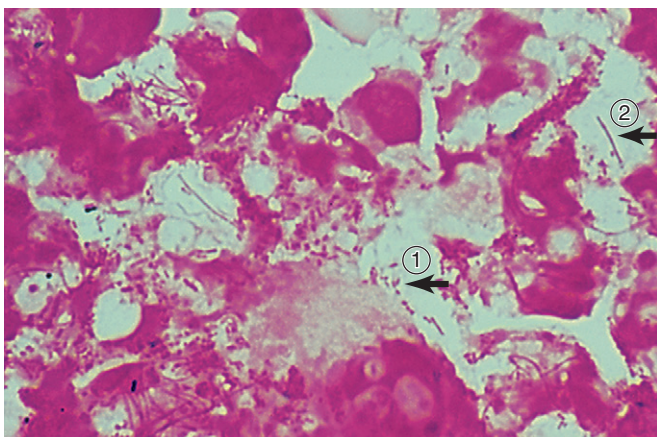


PLATE 40 Amniotic fluid, cytocentrifuge preparation, Gram stain, light microscopy (high-power view). Purulence, moderate. Local materials, moderate. Gram-negative coccobacilli (arrow 1). Gram-negative bacilli, filamentous, medium, fusiform (arrow 2). Morphology suggests gram-negative bacillary anaerobic infection. *Impression:* amnionitis, mixed anaerobic bacteria.

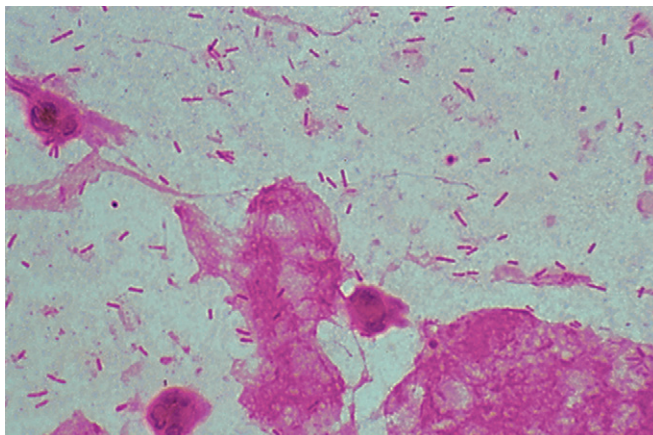


PLATE 43 Expecterated sputum, smear, Gram stain, light microscopy (medium-power view). Purulence, light. Local materials, light. Mucus, moderate. Gram-negative bacilli, medium. *Impression:* enteric bacillary infection.

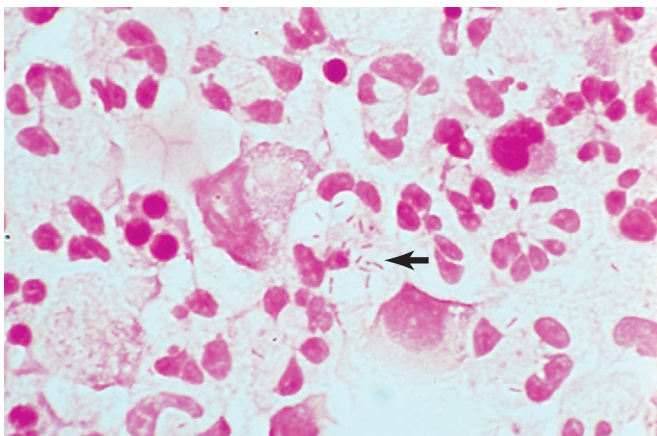


PLATE 41 Bronchoalveolar lavage, cytocentrifuge preparation, Gram stain, light microscopy (high-power view). Purulence, moderate. Local materials, light. Gram-negative bacilli, small, intracellular within phagocytic vacuoles (arrow).

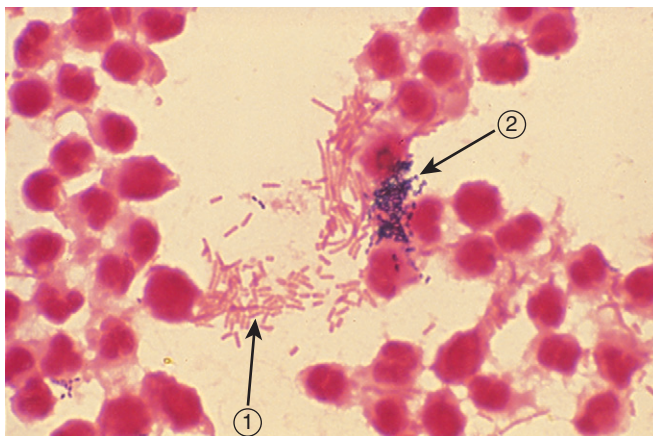


PLATE 44 Urine, direct drop smear, Gram stain, light microscopy (medium-power view). Purulence, heavy. Gram-negative bacilli, medium (arrow 1). Gram-positive cocci (arrow 2). Urine culture grew *Escherichia coli* and *Enterococcus faecalis*. The smear is consistent with a bacterial density of 10^5 colony-forming units per milliliter of urine.

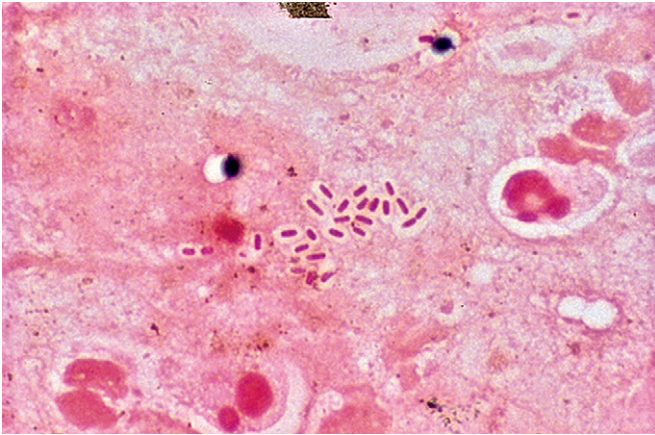


PLATE 45 Decubitus skin ulcer, smear, Gram stain, light microscopy (high-power view). Purulence, moderate. Amorphous debris, heavy. Gram-negative bacilli, medium, encapsulated. Yeast. Morphology suggests *Klebsiella pneumoniae*. Impression: enteric bacillary disease.

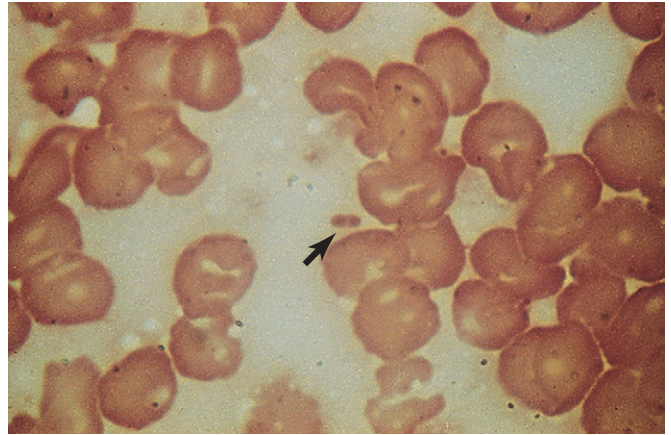


PLATE 48 Peripheral blood, smear, Gram stain, light microscopy (high-power view). Local materials, moderate. Gram-negative bacillus, medium, with prominent bipolar staining (arrow). Suspicious for *Yersinia pestis*. Impression: bubonic plague.

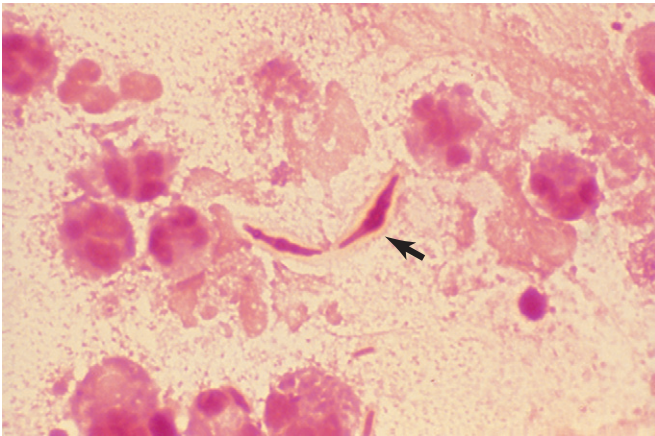


PLATE 46 Expectorated sputum, smear, Gram stain, light microscopy (high-power view). Purulence, moderate. Mucus, moderate. Gram-negative bacilli, aberrant, encapsulated (arrow). Morphology suggests antimicrobial agent-affected *Klebsiella pneumoniae*. Impression: enteric bacillary disease, partially treated.

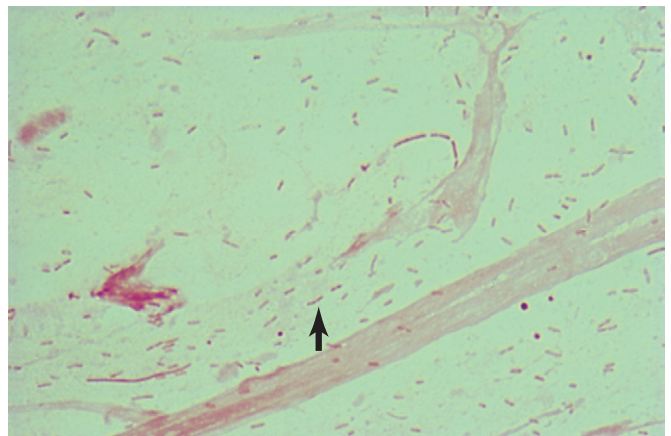


PLATE 49 Expectorated sputum, smear, Gram stain, light microscopy (high-power view). Purulence, none. Local materials, none. Mucus, present. Gram-negative bacilli, regular (arrow). Morphotype suggests *Pseudomonas aeruginosa*. Impression: pseudomonal infectious disease.

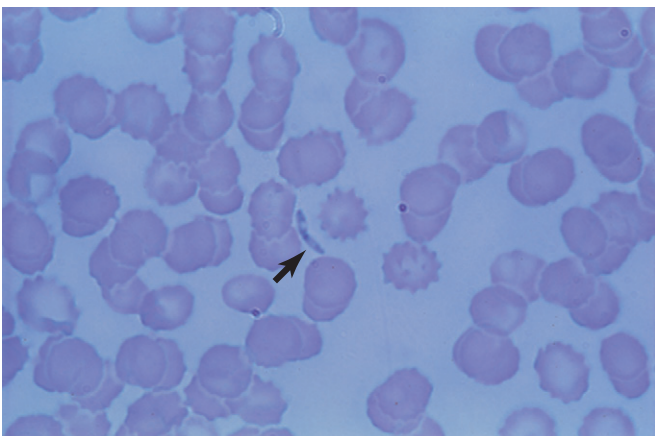


PLATE 47 Peripheral blood, smear, Wright-Giemsa stain, light microscopy (medium-power view). Bacilli, medium, bipolar staining (arrow) (see Plate 48).

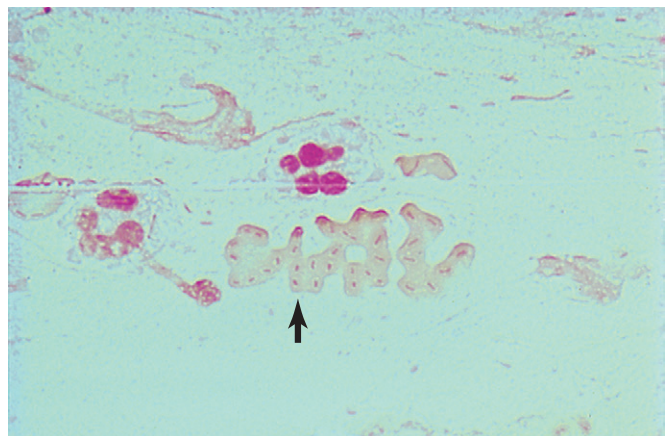


PLATE 50 Expectorated sputum, smear, Gram stain, light microscopy (high-power view). Purulence, light. Local materials, none. Mucus, present. Gram-negative bacilli, regular, enveloped in prominent slime layer (arrow). Morphotype suggests mucoid *P. aeruginosa*. Impression: pseudomonal infectious disease.

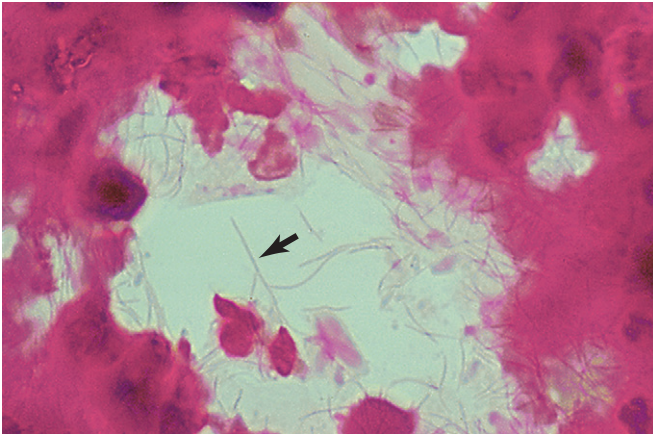


PLATE 51 Amniotic fluid, cytocentrifuge preparation, Gram stain, light microscopy (high-power view). Purulence, light. Local material, moderate. Gram-negative bacilli, fusiform (arrow). Morphology suggests *Fusobacterium* spp. Impression: amnionitis, anaerobic bacteria.

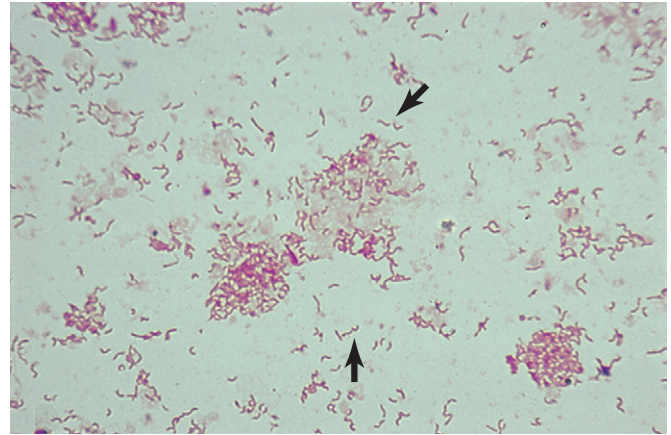


PLATE 53 Colonies on sheep blood agar, subculture from blood culture broth, smear, Gram stain, light microscopy (high-power view). Local material, light. Gram-negative bacilli with spirals, gull wings (arrows). Morphology suggests *Campylobacter* spp.

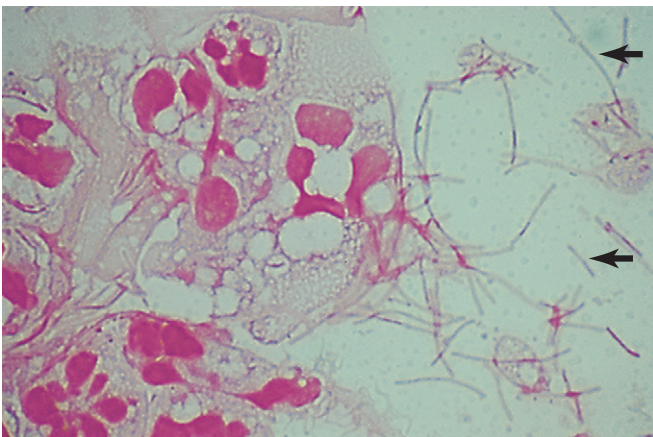


PLATE 52 Amniotic fluid, cytocentrifuge preparation, Gram stain, light microscopy (high-power view). Purulence, moderate. Local material, moderate. Gram-negative bacilli, medium and long forms (arrows). Morphology suggests *Fusobacterium* spp. Impression: amnionitis, anaerobic bacteria.

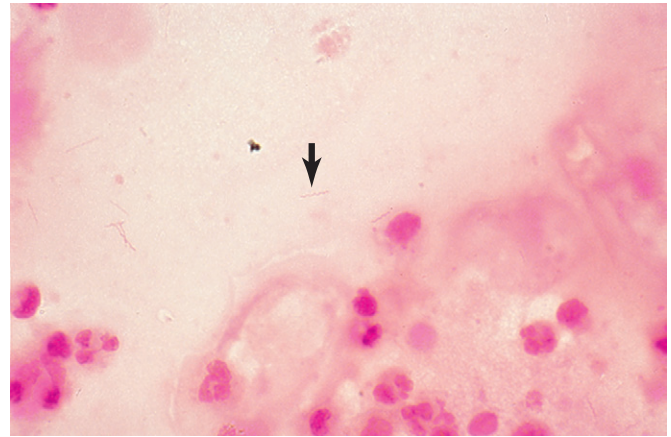


PLATE 54 Amniotic fluid, drop smear, Gram stain, light microscopy (high-power view). Purulence, moderate. Local materials, moderate. Gram-negative bacilli, spiral (arrow). Impression: amnionitis.

Direct examination in polymicrobial infections

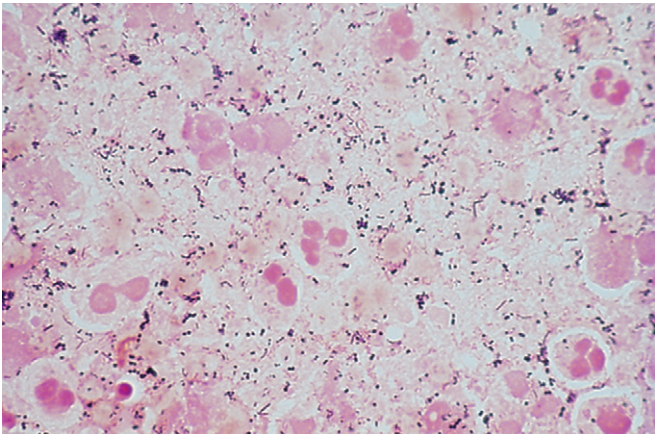


PLATE 55 Buccal space abscess, smear, Gram stain, light microscopy (high-power view). Purulence, heavy. Gram-positive cocci, pairs, chains. Gram-positive bacilli, small, diphtheroid, medium, branched. Gram-negative coccobacilli. Morphology suggests polymicrobial infection from mouth biota. *Impression:* polymicrobial infection, oropharyngeal biota.

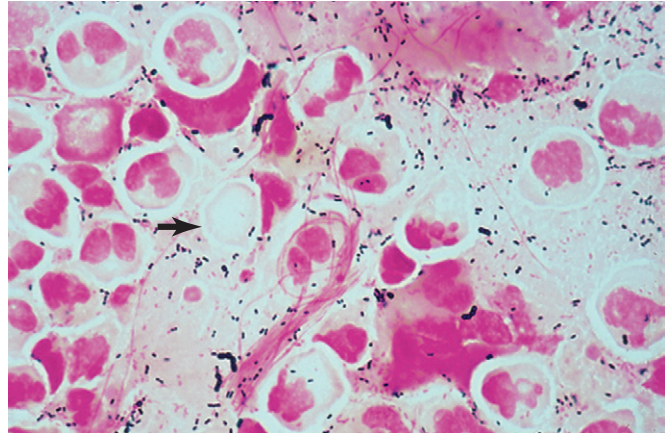


PLATE 57 Cervix, smear, conventional Gram stain, light microscopy (medium-power view). Purulence, heavy. Gram-positive cocci, pairs. Gram-negative coccobacilli. Gram-negative filaments. Trichomonads (arrow, *Trichomonas vaginalis*; compare with Plate 58). *Impression:* trichomoniasis with mixed aerobic and anaerobic bacterial biota.

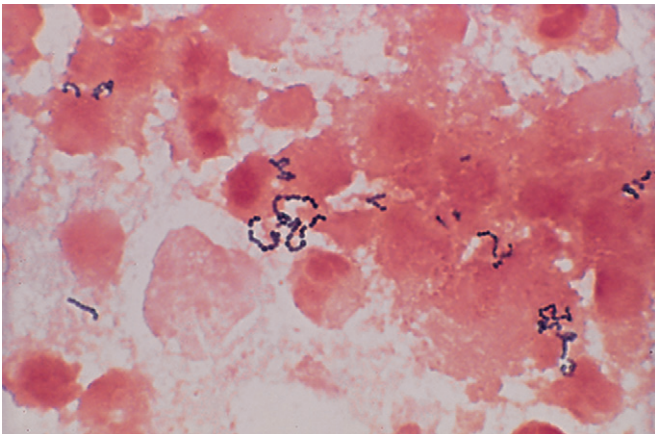


PLATE 56 Maxillary sinus aspirate, smear, Gram stain, light microscopy (high-power view). Purulence, heavy. Gram-positive cocci, chains. Gram-negative coccobacilli, large masses. Morphotype suggests mixed infection with streptococci and anaerobic gram-negative coccobacilli. *Impression:* polymicrobial infection, aerobic and anaerobic species.

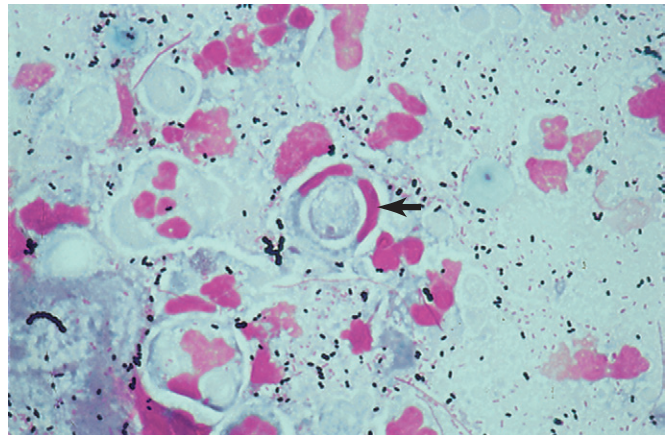


PLATE 58 Cervix, smear, enhanced Gram stain, light microscopy (medium-power view). Purulence, heavy. Gram-positive cocci, pairs. Gram-negative coccobacilli. Gram-negative filaments. Trichomonads (arrow, *Trichomonas vaginalis*). *Impression:* trichomoniasis with mixed aerobic and anaerobic bacterial biota.

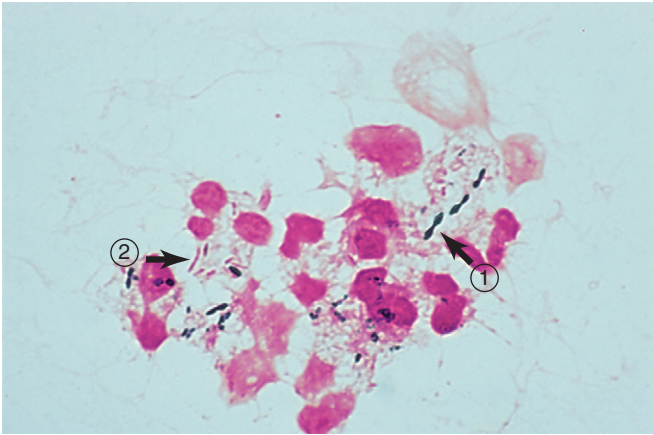


PLATE 59 Eye, vitreous aspirate, smear, Gram stain, light microscopy (medium-power view). Purulence, moderate. Gram-positive diplococci, encapsulated, lancet-shaped (*arrow 1*). Gram-negative bacilli, small, pleomorphic (*arrow 2*). Morphotype suggests mixed infection with *S. pneumoniae* and *H. influenzae*. *Impression*: vitritis, mixed infection.

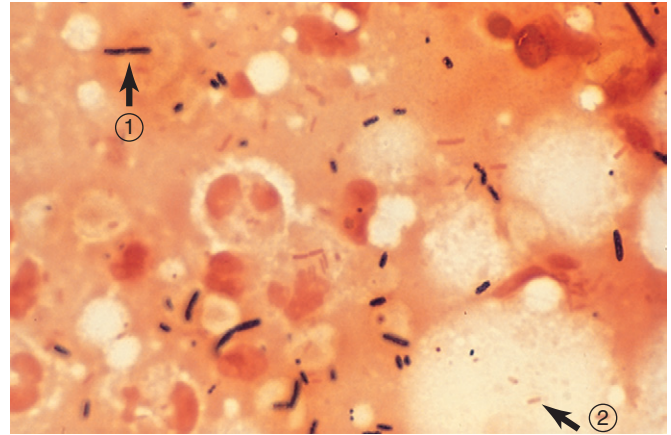


PLATE 61 Drainage, ruptured appendix, smear, light microscopy (high-power view). Purulence, heavy. Gram-positive bacilli, large and medium forms. (*arrow 1*). Gram-negative bacilli (*arrow 2*). Morphology suggests polymicrobial infection with fecal microbiota. *Impression*: polymicrobial infection, fecal biota.

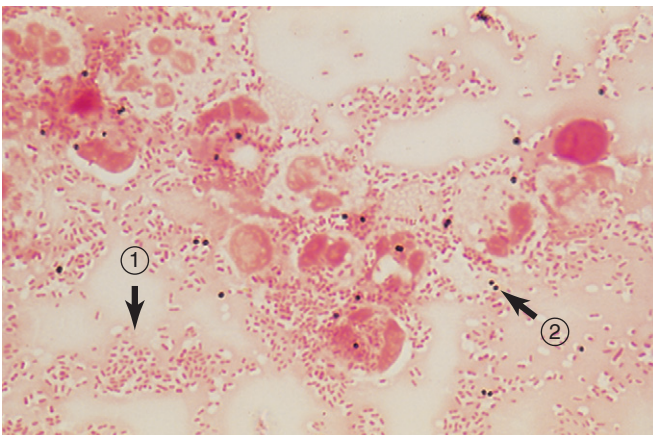


PLATE 60 Wound, smear, Gram stain, light microscopy (medium-power view). Purulence, heavy. Gram-negative bacilli, medium (*arrow 1*). Gram-positive cocci, pairs (*arrow 2*). *Impression*: enteric bacillary infectious disease.

Use of colony morphology for the presumptive identification of microorganisms

Connie R. Mahon

CHAPTER OUTLINE

Importance of colony morphology as a diagnostic tool, 170

Initial observation and interpretation of cultures, 170

Gross colony characteristics used to differentiate and presumptively identify microorganisms, 173

Hemolysis, 173

Size, 174

Form or margin, 174

Elevation, 175

Density, 175

Color, 176

Consistency 176

Pigment 176

Odor 177

Colonies with multiple characteristics, 177

Growth of microorganisms in liquid media, 177

Bibliography, 182

- “Diphtheroids” and staphylococci
- Lactose fermenters and nonlactose fermenters
- “Swarming” *Proteus* species and other members of the order Enterobacterales

KEY TERMS

α-Hemolysis	Lactose fermenter
β-Hemolysis	Margin
Brittle	Nonlactose fermenter
Butyrous	Opaque
Colony morphology	Pigment
Consistency	Rhizoid
Creamy	Smooth
Density	Streamers
Elevation	Swarming
<i>Escherichia/Citrobacter</i>-like organisms	Transillumination
Fastidious	Translucent
Filamentous	Transparent
Hemolysis	Turbidity
<i>Klebsiella/Enterobacter</i>-like organisms	Umbilicate
	Umbonate

OBJECTIVES

After reading and studying this chapter, you should be able to:

1. Describe how growth on blood, chocolate, and MacConkey agars is used in the preliminary identification of bacterial isolates.
2. Differentiate α -hemolysis from β -hemolysis on sheep blood agar medium.
3. Associate the colony characteristics exhibited on blood, chocolate, and MacConkey agars with the microscopic findings on direct smear and use the information in the presumptive identification of microorganisms.
4. Using colony morphology, differentiate among the following microorganisms:
 - Staphylococci and streptococci
 - *Streptococcus agalactiae* and *Streptococcus pyogenes*
 - *Neisseria* spp. and staphylococci
 - Yeasts and staphylococci

Case in point

An exudate from a sacral decubital ulcer on a 65-year-old hospital inpatient was cultured on sheep blood agar (SBA), chocolate (CHOC) agar, and MacConkey (MAC) agar. Direct smear examination showed many white blood cells, a moderate number of gram-positive cocci in pairs and clusters, and a few gram-negative bacilli. After overnight incubation, three colony morphotypes were visible on the SBA and CHOC media. On SBA, the first was a moderate growth of a medium-sized β -hemolytic colony that was off-white with a creamy-buttery texture. The second colony was also β -hemolytic but larger, mucoid, and gray. The third colony type was large, gray, and

mucoid, similar to the second, but was nonhemolytic. The MAC agar showed two colony morphotypes—a light growth of dark pink, dry-looking colonies with a surrounding pink precipitate and a few clear colonies. Based on the Gram stain results and colony characteristics of the isolates, appropriate biochemical tests and antimicrobial susceptibilities were performed to identify the causative agents of the ulcer.

Issues to consider

After reading the patient's case history, consider:

- The direct smear examination findings and how they correlate with the colony morphology of isolates on each culture medium
- The colony morphology of isolates and how it is used to presumptively identify microorganisms
- The colony morphology of each isolate in differentiating between pathogenic and nonpathogenic microorganisms

The importance of the ability to recognize and describe the **colony morphology** (colony characteristics) of microorganisms on culture media and interpretation of Gram-stained smears from clinical specimens cannot be overemphasized. Although Gram-stained smears provide initial identification of microorganisms by microscopic characterization, description of the physical growth characteristics of microorganisms on laboratory media facilitates the initial identification process.

Close your eyes, and imagine the physical characteristics of a person you know well. The person's height, weight, shape, color and style of hair, eyes, freckles, and color of skin as well as voice or laugh may make that person distinctive in a crowd or when his or her back is facing you. In the same manner, many microorganisms have specific characteristics that distinguish them in a crowd of other genera or species.

This chapter discusses the characteristics that are used to describe colony morphology of certain groups of organisms and how these characteristics are used to differentiate one species from a closely related species and one genus from another. It also presents how the characterization of colonies on culture media and the findings on stained direct smears facilitate presumptive identification of commonly isolated organisms.

Importance of colony morphology as a diagnostic tool

The ability of the microbiologist to provide a presumptive identification by colony morphology extends the usefulness of colony characterization beyond a pathway to organism identification and ultimately may include the following:

- **Provide a presumptive identification to the physician.** Even in this age of rapid identification systems, incubation times and procedures can be protracted. In a critical situation, the microbiologist makes an educated judgment about the presumptive identity before performing diagnostic identification procedures.

- **Enhance the quality of patient care through rapid reporting of results and by increasing the cost-effectiveness of laboratory testing.** This may best be illustrated by using sputum cultures as an example. The upper respiratory tract contains many indigenous microorganisms, and to identify every organism in culture would be a time-consuming, cost-prohibitive, and insurmountable task. Microbiologists must be able to differentiate potential pathogens from the “usual” inhabitants (microbiota) of the upper respiratory tract and direct the diagnostic workup toward only potential pathogens. Potential pathogens are presumptively identified by colony characteristics, and preliminary reporting initiates immediate therapy.
- **Play a significant role in quality control, especially of automated procedures and other commercially available identification systems.** When commercial and automated systems are used, a mixed inoculum (polymicrobial, containing more than one species) produces a biochemical test result or erroneous interpretation of reactions that significantly alters the identification (see [Chapter 9](#)). The ability of the microbiologist to determine whether the inoculum is mixed and to ascertain whether the results generated by a commercial or automated system correlate with the suspected identification of the organism is an important component of quality control that is accomplished by recognizing organisms by their colony characteristics.

Initial observation and interpretation of cultures

Primary plating refers to inoculation of the clinical specimen onto laboratory culture media. As previously described in [Chapter 1](#), there are various types of culture media and environmental conditions used in the clinical microbiology laboratory to recover microbial pathogens in clinical samples. The microbiologist must be aware of how each medium functions, the ingredients present, inhibitory agents added in the medium, and possibly growth factors to support and maximize recovery of pathogens. Some laboratory media are selective in that agents are added to inhibit the growth of certain species. This allows better recovery of pathogenic bacteria that are resistant to the inhibitory agents. Some media are differential, permitting the differentiation of bacterial strains based on colony morphology. Differential media are based on the ability of some bacteria, and not others, to utilize a substrate that often produces a change in the pH that is detected by a change in the color of a pH indicator. The ability to determine which organisms grow on selective and differential media aids the microbiologist in making an initial distinction between gram-positive and gram-negative isolates. SBA and CHOC agar, for example, support the growth of various **fastidious** organisms (hard to grow and require additional growth factors) as well as non-fastidious organisms, both gram-positive and gram-negative bacteria.

Clinical microbiology laboratories establish culture plating media protocols that guide microbiologists as to which

media must be inoculated with different clinical specimens (see [Chapter 6](#) for a discussion on specimen collection and processing). For example, specimens such as wounds are plated on culture media such as SBA, CHOC agar, and MAC agar to recover both gram-positive and gram-negative organisms that may be present. Because enteric pathogens are expected in a stool sample, a highly selective medium such as Hektoen enteric is added to MAC agar to enhance recovery of enteric pathogens (e.g., *Salmonella*). [Table 8.1](#) lists some of the media commonly used in a clinical microbiology laboratory along with the key ingredients and their common uses (see [Appendix B on Evolve](#) for a more complete list of laboratory culture media).

Generally, microbiologists observe the colony morphology of organisms isolated on primary culture after 18 to 24 hours of incubation. This process is commonly referred to as *plate reading*, a comparative examination of the colony morphology of microorganisms growing on various culture media. During this process, each type of agar plate

is examined in relationship to the other. As a set of culture media, comparative colony examination of growth from a specimen occurs. To illustrate, generally, organisms that grow on SBA also grow on CHOC agar, but not all organisms that grow on CHOC agar grow on SBA. Although SBA supports fastidious organisms, highly fastidious species, such as *Haemophilus* spp. and *Neisseria gonorrhoeae*, do not grow on it. CHOC agar provides additional nutrients to support highly fastidious organisms such as *Haemophilus* spp. and *N. gonorrhoeae*. Therefore, a gram-negative bacillus that grows on CHOC agar but not on SBA or MAC agar would be suspected to be *Haemophilus* spp., whereas gram-negative diplococci with the same growth pattern would be suspected to be *N. gonorrhoeae* ([Fig. 8.1](#)). The microbiologist is then able to provide a presumptive identification and determine how to proceed in identifying the isolated organisms. Bacterial colonies selected for further testing will be inoculated to other plates, a process referred to as *subculturing*.

Table 8.1 Commonly used media in the clinical microbiology laboratory

Medium	Key ingredients	Uses
Anaerobic blood agar, CDC	Intact sheep red blood cells, vitamin K, yeast extract	Nutritious medium for the isolation and subculturing of obligate anaerobic bacteria
Campy-CVA	<i>Brucella</i> agar base medium; antimicrobials cefoperazone, vancomycin, and amphotericin B; 5% sheep blood agar	Selective for the isolation of <i>Campylobacter</i> spp. in stool specimens
Chocolate agar	GC agar base (provides casein and meat peptones) Hemolyzed sheep red blood cells or supplemented with 2% hemoglobin and NAD or IsoVitaleX (BD BBL, Cockeysville, MD)	Primary plating and subculturing of fastidious bacteria, e.g., <i>Haemophilus</i> and pathogenic <i>Neisseria</i> spp.
Columbia colistin-nalidixic acid (CNA) agar	Columbia agar base, antimicrobials colistin and nalidixic acid 5% sheep red blood cells	Selective isolation of gram-positive cocci, e.g., staphylococci and streptococci
Gram-negative (GN) broth	Peptone-base broth with dextrose (glucose) and mannitol Sodium citrate, and sodium deoxycholate	Selective enrichment broth medium for isolation of gram-negative enteric bacteria, <i>Salmonella</i> and <i>Shigella</i> spp.
Hektoen enteric agar	Lactose, salicin, and sucrose, bile salts to inhibit growth of gram-positive bacteria and many nonpathogenic enteric bacteria	A selective and differential medium for the primary plating of stool specimens to aid in the recovery of intestinal pathogens (e.g., <i>Salmonella</i>)
Lowenstein-Jensen medium	Potato flour, egg and glycerol Asparagine to enhance niacin production Malachite green inhibits growth of other bacteria	Used to cultivate <i>Mycobacterium</i> spp.
MacConkey agar	Lactose and bile salts, the low concentration of bile salts inhibits gram-positive bacteria but permits the growth of many gram-negative bacteria	A selective and differential medium for the isolation of gram-negative bacteria; used for primary plating and subculturing
Modified Thayer-Martin agar	Chocolate agar base containing the antimicrobial agents vancomycin, colistin, trimethoprim, and nystatin	A selective primary plating medium for recovery of <i>Neisseria gonorrhoeae</i> and <i>Neisseria meningitidis</i>
Sabouraud dextrose agar	Dextrose (glucose), antimicrobials can be added to inhibit bacteria	Primary plating and subculturing of fungi
Sheep blood agar	Tryptic soy agar base 5% sheep red blood cells	Primary plating and subculturing of most medically important bacteria
Thioglycollate broth	Contains many nutritive factors, including glucose, casein, yeast, beef extracts, and vitamins Thioglycollate, cystine, and sodium sulfite as reducing agents	Enriched broth medium Supports growth of aerobes, anaerobes, microaerophilic and fastidious organisms

CDC, Centers for Disease Control and Prevention; NAD, nicotinamide adenine dinucleotide.

Case check 8.1

As illustrated in the Case in Point, the Gram-stained smear showed gram-positive cocci and gram-negative bacilli. Three colony morphotypes were observed on SBA; one was identified as gram-positive cocci, and the other were two different gram-negative species. The direct Gram-stained smear will not allow distinctions between the two different gram-negative bacteria. Similar colony morphology observations, except for hemolysis, was made on CHOC agar.

Whereas MAC agar supports most gram-negative rods, especially the Enterobacterales, it inhibits growth of gram-positive organisms and some fastidious gram-negative organisms, such as *Haemophilus* and *Neisseria* spp. Growth on SBA and CHOC agar but not on MAC agar is indicative of a gram-positive isolate or of a fastidious gram-negative bacillus or coccus.

On SBA and CHOC agar, gram-negative rods often produce large colonies that appear gray and sometimes mucoid, and if



Fig. 8.1 Clockwise from top: Chocolate (CHOC) agar plate, sheep blood agar (SBA) plate, and MacConkey (MAC) agar plate. The large colonies growing on all three plates are gram-negative rods (enterics). These gram-negative rods grow larger, gray, and mucoid on SBA and CHOC agar. Notice the smaller, grayish brown fastidious colonies of *Haemophilus* sp. growing on the CHOC agar (arrow), which are not growing on the SBA or MAC agar.

hemolytic, hemolysis is seen on SBA. Because gram-negative rods produce similar-looking colonies on SBA and CHOC agar, they are better described on MAC agar. MAC agar is also best used to characterize gram-negative rods because **lactose fermenters** can be differentiated from those that do not ferment lactose (**nonlactose fermenters**). Lactose fermenters are easily detected by the color change they produce on the medium; as the pH decreases from lactose fermentation, the organisms produce pink, dark pink, or red colonies (Fig. 8.2A). Colonies of nonlactose fermenters remain clear and colorless (see Fig. 8.2B).

The differentiation of lactose fermentation and nonfermentation is particularly important in screening stool cultures. Most enteric pathogens do not ferment lactose, whereas many bacteria that make up the normal microbiota of the intestines do ferment lactose. Microorganisms grow on culture media in the same proportion or concentration in which they are present in the clinical specimen. Because many specimens are polymicrobial, this feature can be beneficial in identifying different colony types.

The microbiologist must also be aware of factors that can significantly alter the colony morphology of growing microorganisms. Incubation time may differ according to when the specimen is received and processed in the laboratory, and this may affect the typical morphology of a certain isolate. For example, young cultures of *Staphylococcus aureus* may appear smaller and might not show the distinct β -hemolysis that older cultures produce. Therefore it is important that growth on primary culture is observed in no less than 18 hours and no longer than 24 hours of incubation.

Case check 8.2

Certain enteric pathogens, such as ***Escherichia/Citrobacter*-like organisms**, produce dry, pink colonies with a surrounding “halo” of pink, precipitated bile salts (Fig. 8.3), whereas ***Klebsiella/Enterobacter*-like organisms** produce large, mucoid pink colonies that occasionally have cream-colored centers (Fig. 8.4). These characteristics on MAC agar are helpful in presumptive identification. In the Case in Point, dry, dark pink colonies were observed on MAC agar, indicating the presence of a lactose-fermenting, gram-negative rod. Colonies of nonlactose fermenters that were clear and colorless (see Fig. 8.2B) were also recovered from this patient’s sample.

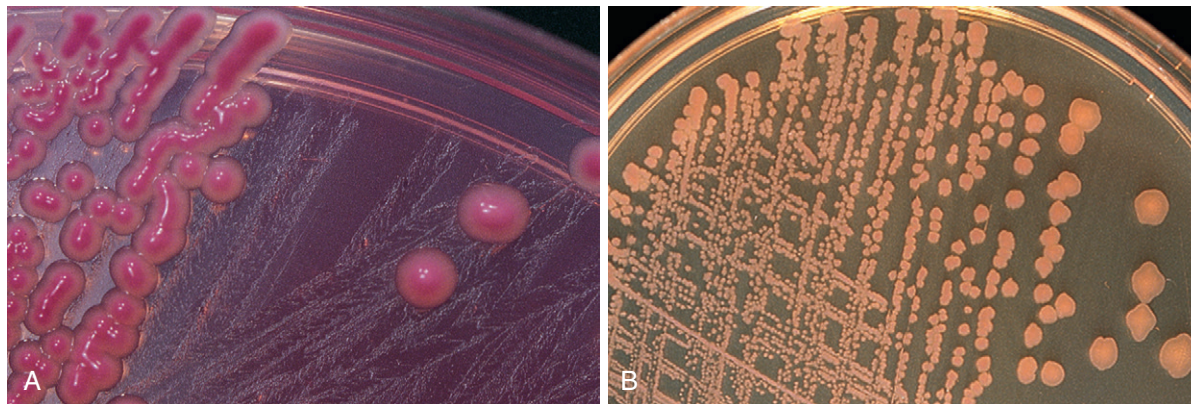


Fig. 8.2 A, Lactose-fermenting, gram-negative rods producing pink colonies on MacConkey (MAC) agar. B, Nonlactose fermenter, gram-negative rods producing colorless colonies on MAC agar.

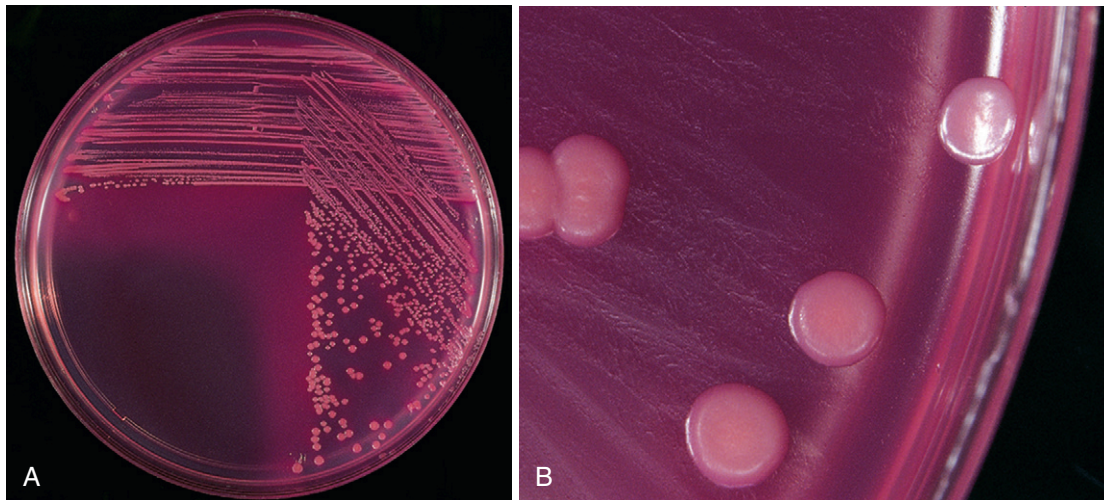


Fig. 8.3 **A**, Lactose-fermenting *Escherichia/Citrobacter*-like organisms growing on MacConkey (MAC) agar. Notice the dry appearance of the colony and the pink precipitate of bile salts extending beyond the periphery of the colonies. **B**, Close-up of dry, flat *Escherichia/Citrobacter*-like lactose fermenters growing on MAC agar. Compare with Fig. 8.4B.

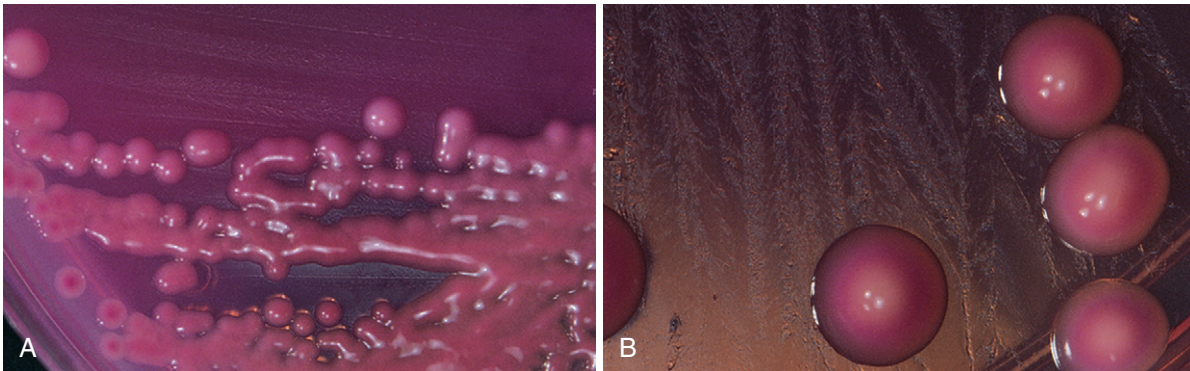


Fig. 8.4 **A**, *Klebsiella/Enterobacter*-like lactose fermenters growing on MacConkey (MAC) agar. Notice the pink, heaped, mucoid appearance. **B**, Close-up of *Klebsiella/Enterobacter*-like colonies on MAC agar. Notice the mucoid, heaped appearance and the slightly cream-colored center after 48 hours of growth.

Gross colony characteristics used to differentiate and presumptively identify microorganisms

By observing the colony characteristics of the organisms that have been isolated, the microbiologist is able to make an “educated guess” regarding the identification of the isolate. The following descriptions are routinely used to examine colony characteristics. Many of the following colony characteristics may differ among species and strains of the same genus.

Hemolysis

Hemolysis (Greek *hemo*: pertaining to red blood cells [RBCs]; *lysis*: dissolution or break apart) is a reaction caused by enzymatic or toxin activity of bacteria. Hemolysis, observed on SBA seen immediately surrounding or underneath the colony (e.g., α , β , or no hemolysis) with other colony characteristics, is helpful in presumptive identification, particularly of streptococci and enterococci (see Chapter 15). Proper technique

requires the passing of bright light through the bottom of the plate (**transillumination**) to determine whether the organism is hemolytic (Fig. 8.5). Occasionally, the colony must be removed with a loop to visualize the hemolytic pattern. It is important to determine whether true hemolysis is present or whether the discoloration of the medium is the result of growth of the organism on the plate. Organisms that have no lytic effect on the RBCs in SBA are referred to as *nonhemolytic*.

α -Hemolysis

α -Hemolysis is partial lysing of RBCs in an SBA plate around and under the colony that results in a green discoloration of the medium. Examples of organisms that produce α -hemolysis include *Streptococcus pneumoniae* and certain viridans streptococci (see Fig. 8.24 [later] for a comparison of the colony morphology of these organisms).

β -Hemolysis

β -Hemolysis is complete clearing of erythrocytes in SBA around or under the colonies because of the complete lysis of RBCs.

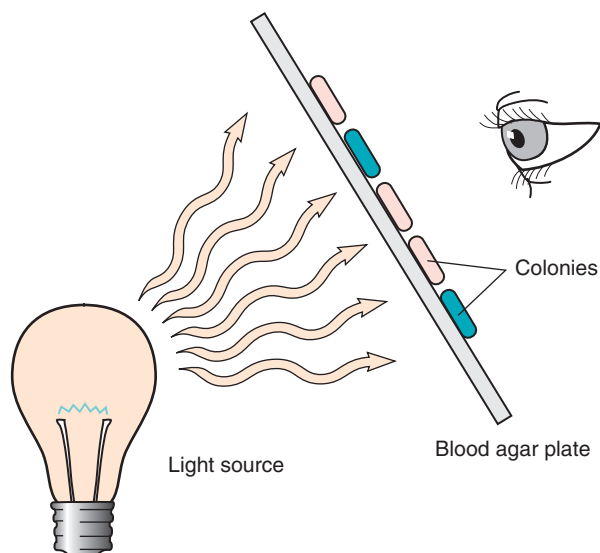


Fig. 8.5 The use of transillumination to determine whether colonies are hemolytic. The technique can be used for MacConkey agar also to see slight color differences in nonlactose fermenters.

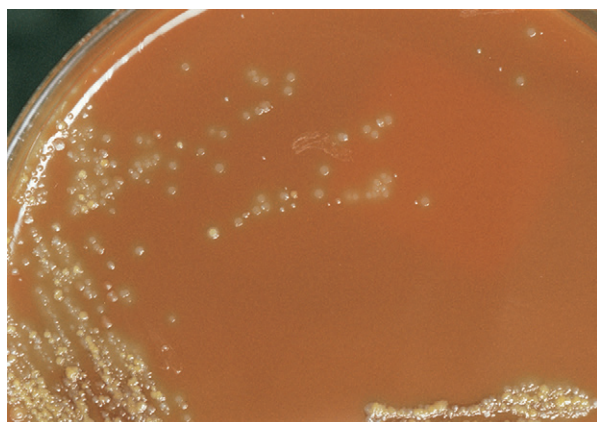


Fig. 8.6 Chocolate (CHOC) agar does not display hemolysis because the red cells in the medium have already been lysed. Bacteria that are hemolytic on sheep blood agar usually have a green coloration around the colony on CHOC agar.

Certain organisms, such as group A β -hemolytic streptococci (*Streptococcus pyogenes*), produce a wide, deep, clear zone of β -hemolysis, whereas others, such as group B β -hemolytic streptococci (*Streptococcus agalactiae*) and *Listeria monocytogenes* (a gram-positive rod) produce a narrow, diffuse zone of β -hemolysis close to the colony. These features are helpful hints in the identification of certain bacterial species (see Fig. 8.25 [later] for a comparison of the colony characteristics of group A and group B streptococci). CHOC agar does not display hemolysis because the RBCs in the medium were lysed during media preparation. Organisms that are α -hemolytic or β -hemolytic on SBA usually show a green coloration around the colony on CHOC agar (Fig. 8.6). However, this coloration should not be mistaken for a hemolytic characteristic.

Size

Colonies are described as large, medium, small, or pinpoint. However, a microbiologist rarely measures a colony with



Fig. 8.7 Left, sheep blood agar (SBA) plate: small, white colonies are gram-positive cocci. Right, SBA plate: large, gray, mucoid colonies are enteric gram-negative rods.

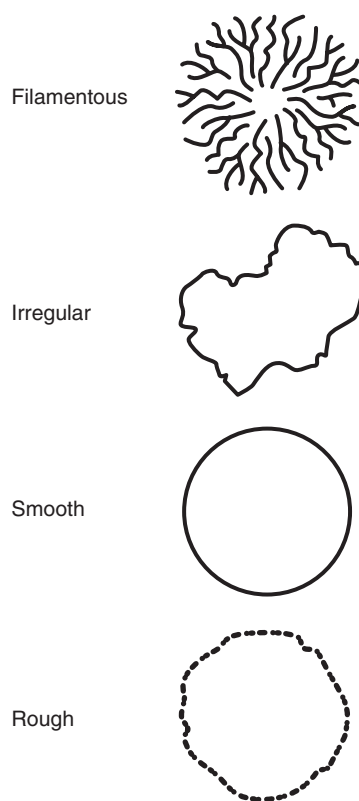


Fig. 8.8 Illustration of form or margin to describe colony morphology.

a ruler. Size is generally a visual comparison between genera or species. For example, gram-positive bacteria generally produce smaller colonies than gram-negative bacteria. *Staphylococcus* species are usually larger than *Streptococcus* species. Fig. 8.7 shows colonies of gram-negative rods compared with gram-positive cocci.

Form or margin

The edge of the colonies should be observed and the form, or **margin**, described as **smooth**, **filamentous**, rough, **rhizoid**, or irregular (Fig. 8.8). Colonies of *Bacillus anthracis* on visual

examination are described as “Medusa heads” because of their filamentous appearance. Certain genera such as *Proteus* (especially *Proteus mirabilis*) may swarm on nonselective agar such as SBA or CHOC agar. **Swarming** is a hazy blanket of growth on the surface that extends well beyond the streak lines. Fig. 8.9 shows swarming colonies of *Proteus* spp. Diphtheroids produce colonies that have a dry appearance (Fig. 8.10). Certain yeasts produce colonies that are creamy white with a dull surface and are described as colonies with feet or pedicles, whereas staphylococci produce moist, creamy white to yellowish colonies (see Fig. 8.26 [later] for a comparison of the colony morphology of yeasts and staphylococci).

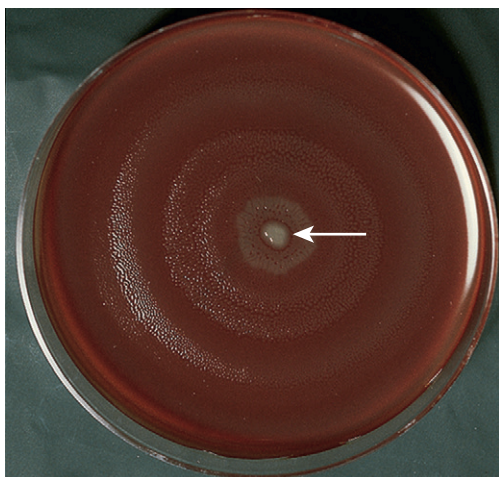


Fig. 8.9 Swarming colonies of *Proteus* spp. The organism was inoculated in the middle of the sheep blood agar plate (arrow).

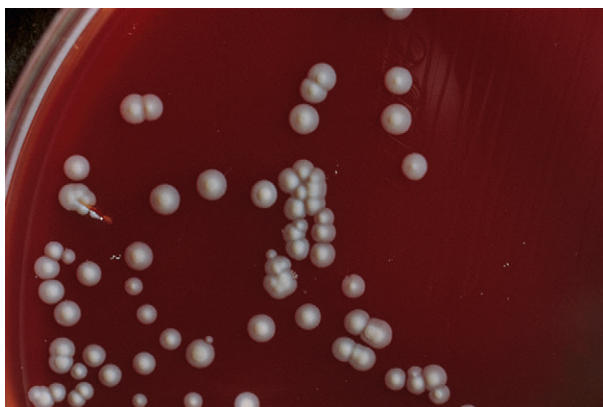


Fig. 8.10 “Diphtheroid” colonies with dry appearance and umbonate center growing on sheep blood agar plate.

Elevation

The **elevation** should be determined by tilting the culture plate and looking at the side of the colony (Fig. 8.11). Elevation may be raised, convex, flat, **umbilicate** (depressed center, concave), or **umbonate** (raised or bulging center, convex). *S. pneumoniae* typically produces umbilicate colonies unless the colonies are mucoid because of the presence of a polysaccharide capsule. *S. aureus* typically produces convex colonies. In comparison, β -hemolytic streptococci generally produce flat colonies.

Density

The **density** of the colony can be **transparent**, **translucent**, or **opaque**. To see the differences in the density of colonies, it is useful to look through the colony while using transillumination. Translucent colonies allow some light to pass through the colony, and opaque colonies do not (Fig. 8.12). β -Hemolytic streptococci except group B (*S. agalactiae*) are described as translucent. *S. agalactiae* produces colonies that are semi-opaque, with the organisms concentrated at the center of the colony, sometimes described as a *bull's-eye* colony. Staphylococci and other gram-positive bacteria are usually opaque. Most gram-negative rods also form opaque colonies. *Bordetella pertussis* is described as shiny, similar to a half-pearl, on blood-containing media (see Chapter 18).

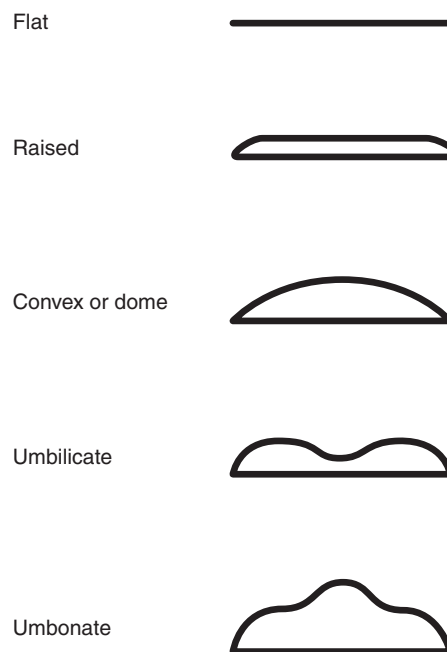


Fig. 8.11 Illustration of elevations to describe colony morphology.

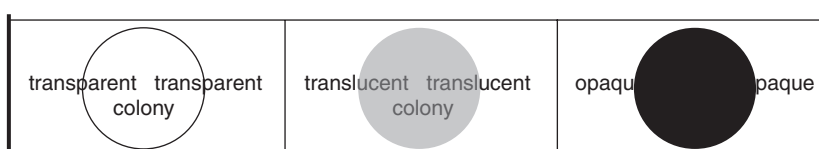


Fig. 8.12 Illustration of density to describe colony morphology.

Color

In contrast with pigmentation, *color* is a term used to describe a particular genus in general. Colonies may be white, gray, yellow, or buff. Coagulase-negative staphylococci are white (Fig. 8.13), whereas *Enterococcus* spp. may appear gray. Certain *Micrococcus* spp. and *Neisseria* spp. (nonpathogenic) are yellow or off-white (Fig. 8.14). Diphtheroids are buff. Most gram-negative rods are gray on SBA.

Consistency

Consistency is determined by touching the colony with a sterile loop. Colony consistency may be **brittle** (splinters), **creamy (butyrous)**, dry, or waxy. Occasionally, the entire colony adheres (sticks) to the loop. As examples, *S. aureus* is creamy, whereas certain *Neisseria* spp. are sticky. *Nocardia* spp. produce colonies that are brittle, crumbly, and wrinkled, resembling bread crumbs on a plate. Diphtheroid colonies are usually dry and waxy. Most β -hemolytic streptococci are dry (except for mucoid types), and when pushed by a loop, the whole colony remains intact.

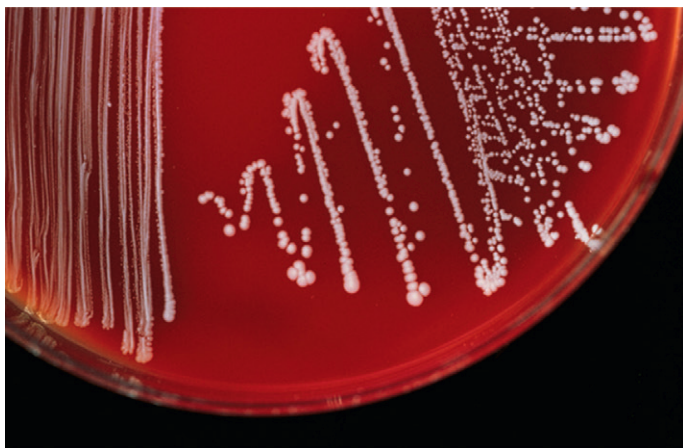


Fig. 8.13 Example of white colonies of coagulase-negative staphylococci on sheep blood agar plate.

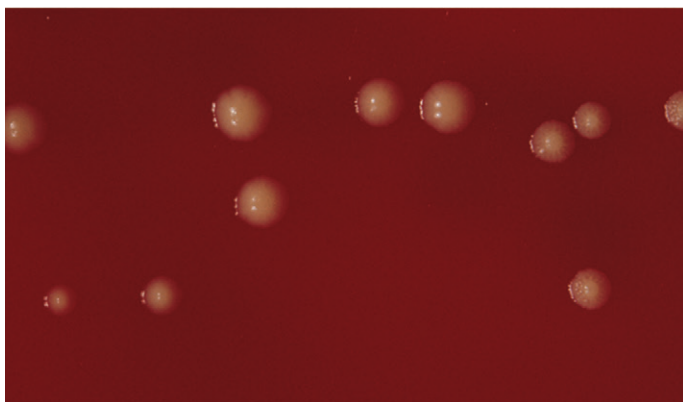


Fig. 8.14 Example of the yellow colonies characteristic of certain nonpathogenic *Neisseria* spp. on sheep blood agar plate.

Pigment

Pigment production is an inherent characteristic of a specific organism confined generally to the colony, although some pigments will diffuse through the culture medium. Pigment production is generally enhanced by growing bacteria at room temperature. Examples of organisms that produce pigment include the following:

- *P. aeruginosa*—green, sometimes a metallic sheen (Fig. 8.15)
- *Serratia marcescens*—brick red (Fig. 8.16), especially at room temperature
- *Kluyvera* spp.—blue
- *Chromobacterium violaceum*—purple
- *Prevotella melaninogenica*—brown-black (anaerobic)

Pigment production for these organisms is variable.

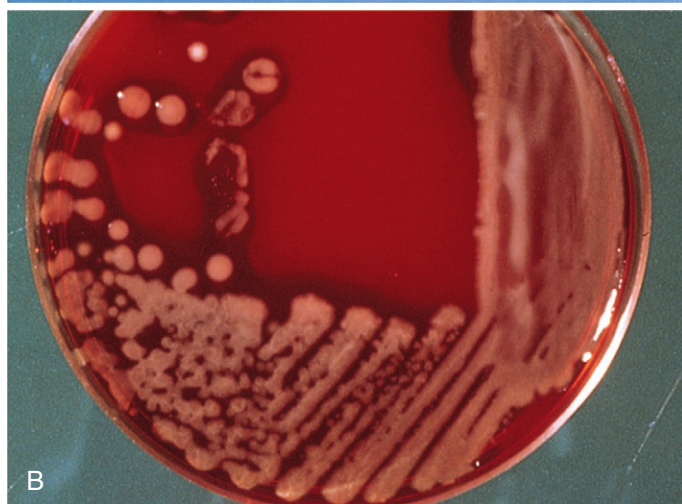
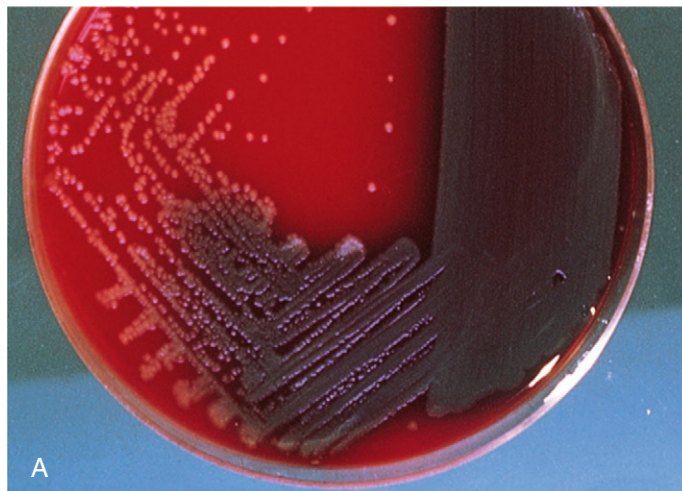


Fig. 8.15 **A**, *Pseudomonas aeruginosa* illustrating the metallic sheen and green pigmentation of colonies on sheep blood agar (SBA) plate. **B**, Not all strains of this organism have the same colony appearance. This is a mucoid strain of *P. aeruginosa* on SBA.

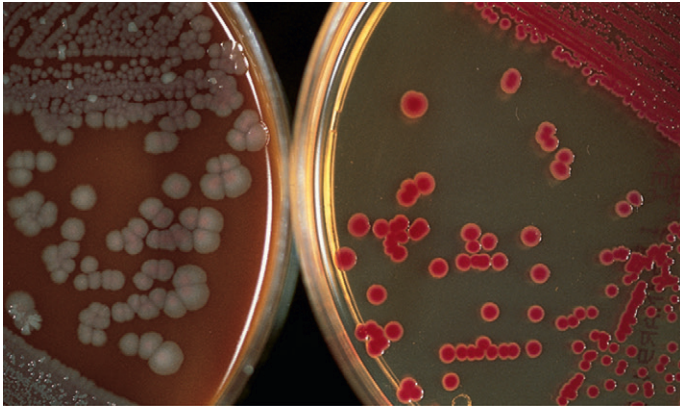


Fig. 8.16 Brick-red pigment of *Serratia marcescens* that is evident on MacConkey agar (right). This brick-red pigment should not be confused with lactose fermentation. The pigment is slightly visible on a chocolate agar plate (left). Incubation at room temperature enhances pigmentation.

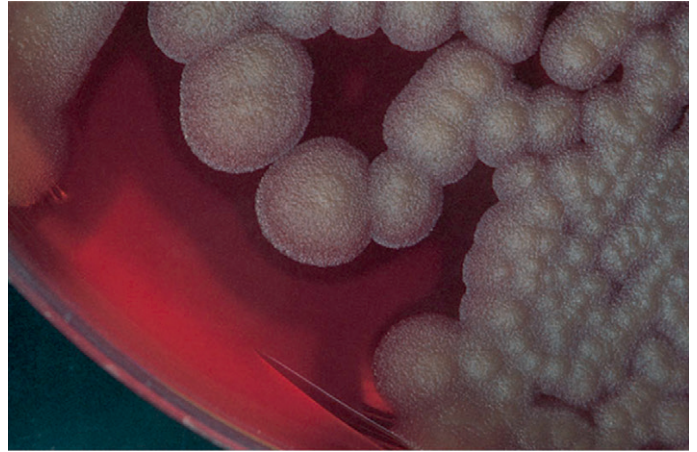


Fig. 8.17 Large, rough, hemolytic colonies of *Bacillus cereus* on sheep blood agar plate.

Odor

Although certain organisms produce distinctive odors, the microbiologist should never inhale directly from the plate. Odor should be determined when the lid of the culture plate is removed, and the odor dissipates into the surrounding environment. Examples of microorganisms that produce distinctive odors are as follows:

- *S. aureus*—old sock (stocking that has been worn continuously for a few days without washing); this odor is evident when bacteria are growing on mannitol salt agar
- *P. aeruginosa*—fruity or grapelike
- *P. mirabilis*—putrid
- *Haemophilus* spp.—musty basement, “mousy” or “mouse nest” smell
- *Nocardia* spp.—freshly plowed field

Case check 8.3

The Case in Point illustrates the deductive reasoning that occurs during plate reading (colony morphology) of the culture and examination of the direct smear of the clinical specimen. Both techniques have an important role in the presumptive identification required in plate reading. The first step is to examine the direct smear of the specimen for important clues, for example, the presence of white blood cells (an inflammatory process) and specific Gram stain morphology. Gram-positive cocci in pairs and clusters in the direct smear are suggestive of staphylococci (see Chapter 7); it is difficult to distinguish among enteric gram-negative bacilli. The β -hemolytic, white, or off-white, creamy butter-looking, medium colonies on SBA are highly suggestive of *S. aureus* (see Fig. 8.26B). *S. aureus* would be inhibited by MAC agar and would not grow, leaving the other two colony types to identify. The lactose fermenter (pink) on MAC agar with a halo of pink precipitate surrounding the colonies is indicative of *Escherichia/Citrobacter*-like organisms. Of these two, *Escherichia coli* can be β -hemolytic on SBA (see Chapter 19). The nonlactose fermenter is the third type of colony present in the clinical specimen. Both lactose fermenters and nonlactose fermenters are growing on SBA because this medium is noninhibitory but are best differentiated on MAC agar.

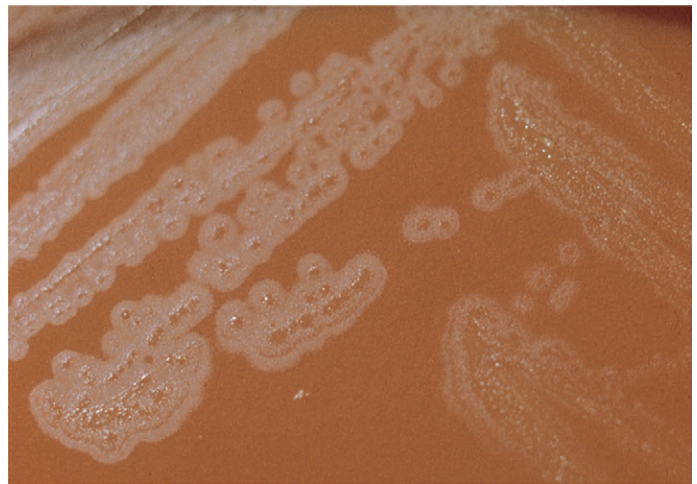


Fig. 8.18 Small, “fuzzy-edged,” umbonate center colony of *Eikenella corrodens* on chocolate agar. This organism has the tendency to “pit” the agar.

Colonies with multiple characteristics

In addition to the organisms already mentioned, other bacteria fit in multiple descriptive categories of colony morphology. *Bacillus cereus* forms large, rough, greenish, hemolytic colonies on SBA (Fig. 8.17). *Eikenella corrodens* forms a small, “fuzzy-edged” colony with an umbonate center on SBA or CHOC agar (Fig. 8.18). Roughly one half of the strains will corrode or form pits in the agar.

Growth of microorganisms in liquid media

Important clues to the identification of an organism can also be detected by observing the growth of the organism in liquid media such as thioglycollate. **Streamers** or vines and puffballs are associated with certain species of streptococci (Fig. 8.19). **Turbidity**, which refers to cloudiness of

the medium resulting from growth (and usually gas if the medium contains glucose), is produced by many Enterobacterales (Fig. 8.20). Yeasts and *Pseudomonas* species produce scum at the top and sides of the tube (Figs. 8.21 and 8.22). In addition, yeasts occasionally grow below the surface in the microaerophilic area of the medium (Fig. 8.23).

This chapter discussed the importance of recognizing colony morphology in the initial identification of microorganisms. Fig. 8.24A–B (*S. pneumoniae* and α -hemolytic streptococci),

Fig. 8.24C (*Enterococcus* spp.), Fig. 8.25 (*S. pyogenes* and *S. agalactiae*), and Fig. 8.26 (staphylococci and a yeast) show the differences between various organisms by colony morphology. Microbiologists might become frustrated when microorganisms that produce known characteristic features show atypical results in colony morphology, microscopic morphology, and biochemical reactions. Organisms may exhibit characteristics far different from those previously described for them. The ability to recognize these differences and changes in characteristics makes this discipline a challenge.

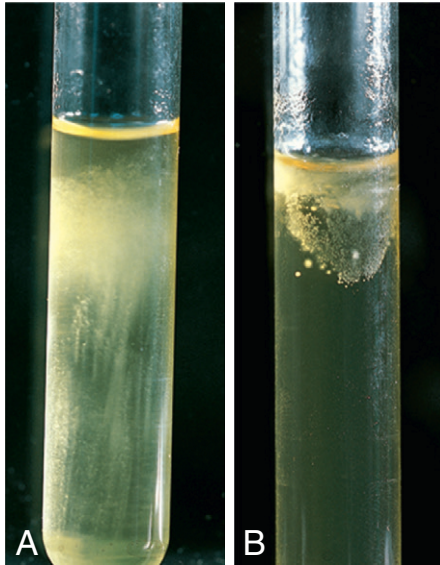


Fig. 8.19 A, “Vine” or “streamer” effect exhibited by certain species of streptococci when growing in thioglycollate broth. The effect is more prevalent toward the bottom of the tube. B, “Puffed balls” effect exhibited by certain streptococcal species when growing in thioglycollate.

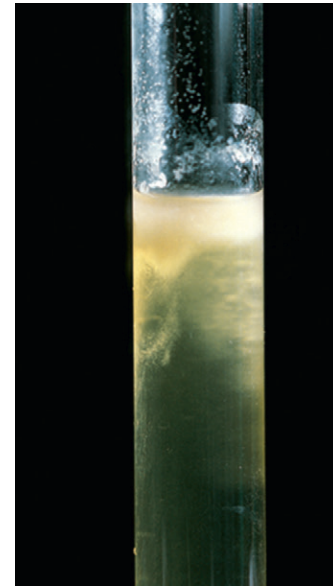


Fig. 8.21 Production of “scum” by a yeast at the surface of thioglycollate broth.

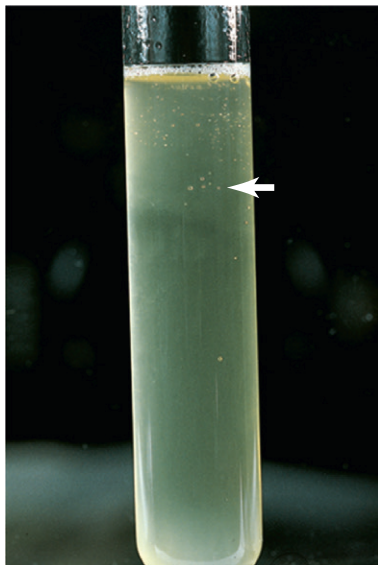


Fig. 8.20 Turbidity produced by enterics when growing in thioglycollate broth. Notice the gas bubbles at the surface of and in the middle of the medium (arrow).

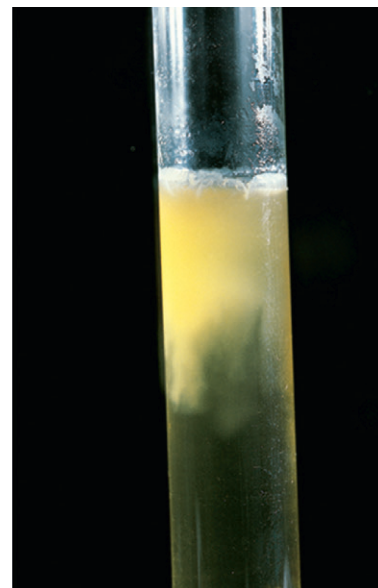


Fig. 8.22 Illustration of *Pseudomonas* sp. producing surface “scum” at the sides of thioglycollate broth. Occasionally, *Pseudomonas aeruginosa* produces a diffusible green pigment and a metallic sheen at the surface.

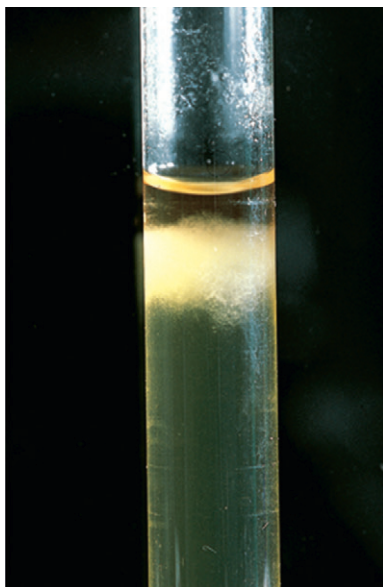


Fig. 8.23 Yeast growing in the microaerophilic area of thioglycollate broth.

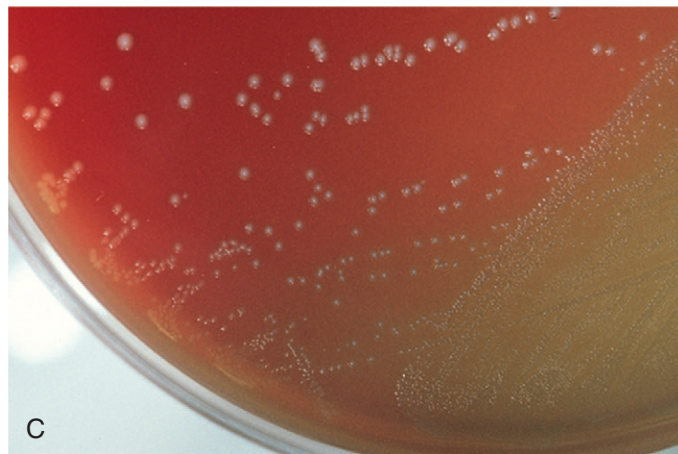
Differentiation of *Streptococcus pneumoniae*, α -hemolytic viridans streptococci, and *Enterococcus* by colonial morphology

<i>Streptococcus pneumoniae</i>	α -Hemolytic viridans streptococci
Translucent, may resemble a water droplet; umbilicate, or flat with "penny" edge; entire margin, wide and strong zone of α-hemolysis  Umbilicate  "Penny" edge	Translucent, grayer, rough margin, umbonate center   Umbonate center

A



B



C

Fig. 8.24 **A**, Differentiation of *Streptococcus pneumoniae* and α -hemolytic viridans streptococci by colony morphology. **B**, *S. pneumoniae* growing on sheep blood agar (SBA). Notice the strong zone of α -hemolysis, umbilicate center, and wet (mucoid) appearance of the colonies. **C**, *Enterococcus* sp. growing on SBA. It does not have an umbilicate or umbonate center, but it is more heaped and gray-appearing than *S. pneumoniae*. Enterococci form larger colonies and a smooth, darker margin, in contrast with many strains of α -hemolytic streptococci. The green color on the plate is not hemolysis but is a discoloration characteristic of growth.

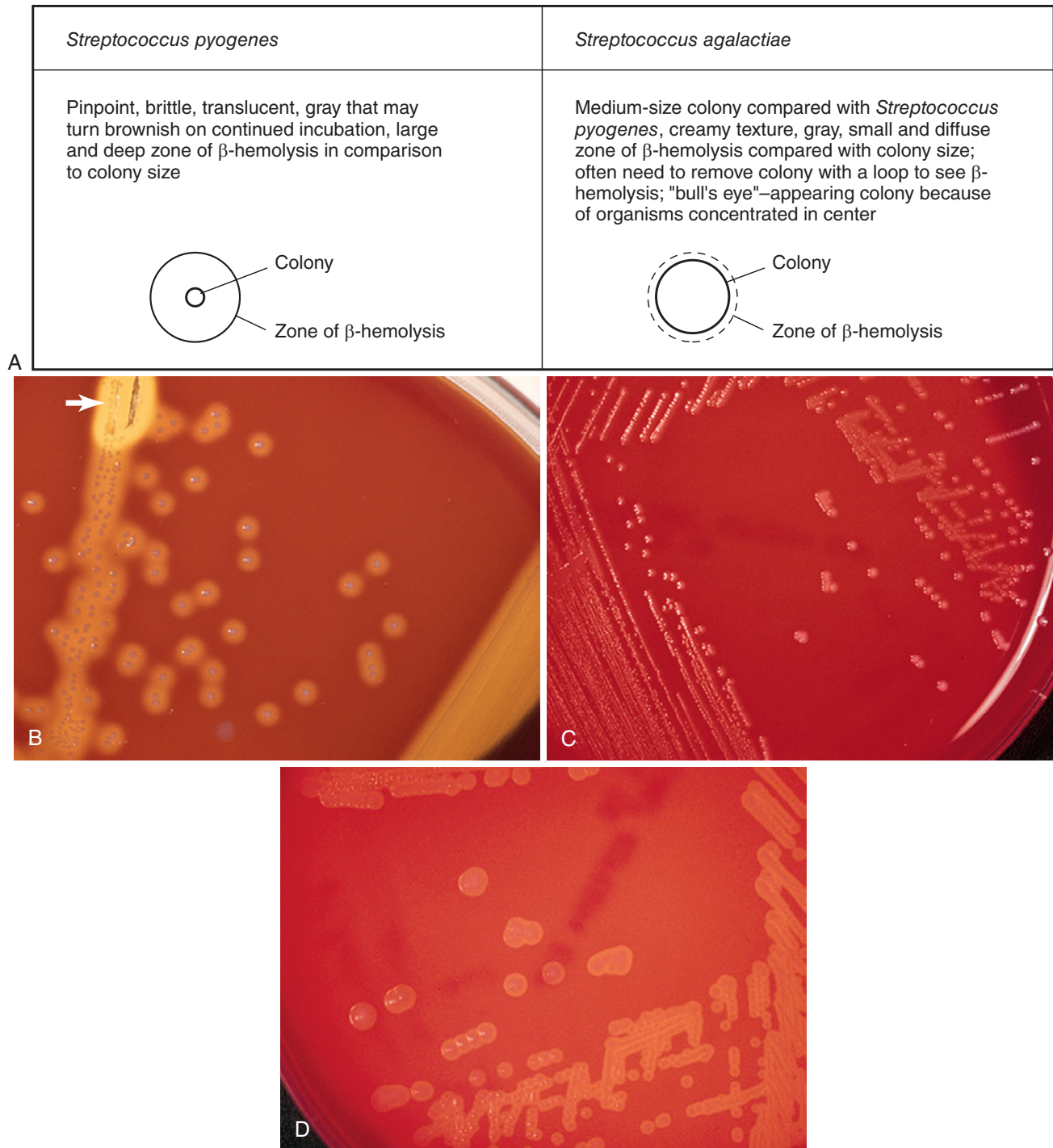


Fig. 8.25 **A**, Differentiation of *Streptococcus pyogenes* and *Streptococcus agalactiae* by colony morphology. **B**, Tiny colony of *S. pyogenes* exhibiting large, deep zone of β -hemolysis on sheep blood agar (SBA). **C**, Colonies of *S. agalactiae* growing on SBA. This organism produces a larger colony and a smaller, more diffuse zone of hemolysis than *S. pyogenes*. The hemolysis is not evident in this photograph. Compare with **B**. **D**, Colonies of *S. agalactiae* growing on SBA. Through the use of transillumination, the hemolytic pattern is now evident; hemolysis is diffuse, and it remains close to the periphery of the colony. The same colony morphology is produced by *Listeria monocytogenes*, a gram-positive rod. Compare with **B**. *S. pyogenes* (arrow) produces two hemolysins; one is oxygen stable, and the other is oxygen labile. Stabbing the medium with an inoculating loop carries the organism into areas where anaerobic conditions are more prevalent, allowing the enhanced hemolysis (due to the oxygen-labile hemolysin) to be seen.

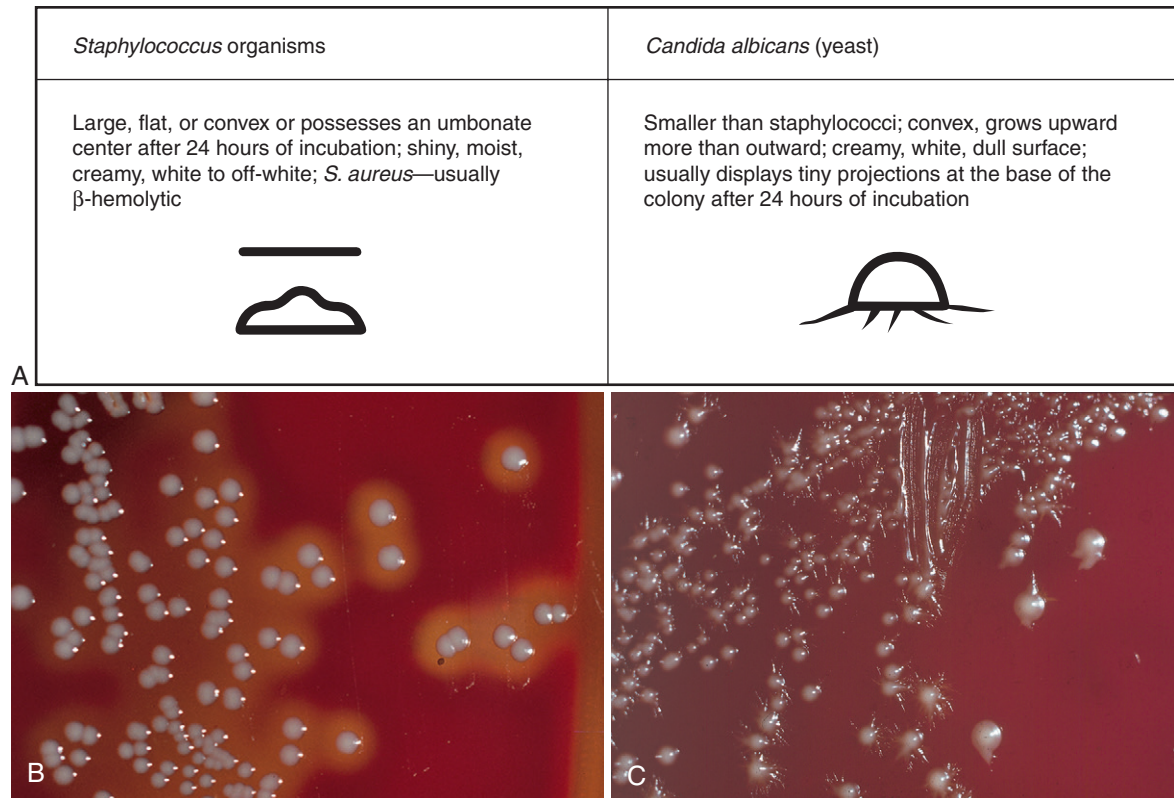


Fig. 8.26 **A**, Differentiation between staphylococci and *Candida albicans* (a yeast) by colony morphology. **B**, Large, white, convex, shiny, moist, β -hemolytic colonies of *Staphylococcus aureus* growing on sheep blood agar (SBA) plate. Most other *Staphylococcus* spp. are nonhemolytic. **C**, “Heaped” or convex, white, dull appearance and butyrous texture of *C. albicans* on SBA. Notice the tiny projections or “feet” at the edge of the colonies.

POINTS TO REMEMBER

- The initial identification process starts with the microscopic morphology on Gram-stained smears and colony morphology of the isolate. Biochemical reactions or molecular biology assays confirm the identification.
- Gram stain of the colony from the culture plate may look different from the direct smear from the specimen. Competition, crowding, and metabolic by-products can alter the Gram stain morphology. For example, in contrast with the direct smear or liquid media, streptococci from the colony may not appear as gram-positive cocci in chains.
- The colony morphology described in this chapter is not infallible. Variations occur quite frequently. The morphologies described are general characteristics for any given organism.

LEARNING ASSESSMENT QUESTIONS

1. What do dark pink colonies on MAC agar indicate? The bacteria are:
 - a. Nonlactose fermenters
 - b. Lactose fermenters
 - c. Gram-positive
 - d. Nonviable
2. In the Case in Point, why are there three colony types that grow on SBA but only two that grow on MAC agar?
3. Which of the following potential pathogens would you suspect if you were to find α -hemolytic colonies from a respiratory sample?
 - a. *Streptococcus pneumoniae*
 - b. *Streptococcus pyogenes*
 - c. Viridans streptococci
 - d. *Haemophilus influenzae*
4. How would you describe the colonies produced on MAC agar by nonlactose fermenting gram-negative bacilli?
5. How would you differentiate β -hemolysis from α -hemolysis?
6. What would you suspect if you noticed “puffballs” growing in broth media?
7. Which genus of bacteria shows “swarming” colonies as a colony characteristic?
 - a. *Klebsiella*
 - b. *Citrobacter*
 - c. *Proteus*
 - d. *Salmonella*
8. A moderate growth of a heaped, dry-appearing, white colony is isolated from a patient suspected of having thrush. The colony has tiny projections extending out along the edge of its margin. Which of the following is a presumptive identification of this organism?
 - a. *Staphylococcus aureus*
 - b. *Staphylococcus epidermidis*
 - c. *Neisseria* spp.
 - d. *Candida albicans*

9. A vaginal culture from a 25-year-old pregnant patient produced colonies on SBA and CHOC agar, but there was no growth on MAC agar. The colonies are described as medium size with small, diffuse zones of β -hemolysis. This type of hemolysis is noticed when a colony is removed with a loop. Which of the following is the suspected identification of this organism?
- Streptococcus pyogenes* (group A)
 - Streptococcus agalactiae* (group B)
 - Streptococcus pneumoniae*
 - Staphylococcus aureus*
10. Which of the following is the reason why hemolysis should not be determined on CHOC agar?
- CHOC is a nonselective agar.
 - Growth factors added on CHOC inhibit hemolysis.
 - Red blood cells in CHOC agar are already lysed.
 - CHOC agar does not support growth of hemolytic organisms.

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Biochemical identification of gram-negative bacteria

Donald C. Lehman

CHAPTER OUTLINE

Carbohydrate utilization, 184

- Oxidation-fermentation tests, 185
- Triple sugar iron agar, 186
- Ortho-nitrophenyl- β -D-galactopyranoside test, 187

Glucose metabolism and its metabolic products, 187

- Methyl red test, 187
- Voges-proskauer test, 187

Amino acid utilization, 188

- Decarboxylase and dihydrolase tests, 188
- Deaminase test, 189

Miscellaneous tests, 189

- Citrate utilization, 189
- Deoxyribonuclease (DNase) production, 190
- Indole production, 190
- Lysine iron agar slant, 190
- Motility, 191
- Motility-indole-ornithine agar, 191
- Nitrate and nitrite reduction, 191
- Oxidase, 191
- Sulfide-indole-motility agar, 191
- Urease, 191

Manual multitest systems, 192

- Principles of identification, 192
- Analytical profile index, 192

Rapid and automated identification systems, 193

- The term *rapid*, 193
- Rapid biochemical tests performed on isolated colonies, 194
- Identification systems relying on carbohydrate utilization or chromogenic substrates, 194
- Automated identification systems, 195
- Evaluation of identification systems, 197

Bibliography, 198

OBJECTIVES

After reading and studying this chapter, you should be able to:

1. Explain the difference between phenotyping and genotyping.
2. Discuss the utilization of lactose by bacteria.
3. Compare the differences between oxidation and fermentation.
4. Explain the different reactions that may be observed in triple sugar iron (TSI) agar.
5. Describe the reactions involved and the products of metabolism tested in each of the following:
 - Ortho-nitrophenyl- β -D-galactopyranoside test
 - Methyl red (MR) and Voges-Proskauer (VP) test
 - Decarboxylase, dihydrolase, and deaminase tests
 - Citrate utilization test
 - Deoxyribonuclease (DNase) test
 - Gelatin liquefaction test
 - Indole test
 - Nitrate and nitrite reduction tests
 - Oxidase test
 - Urease test
 - Lysine iron agar (LIA) test
 - Motility-indole-ornithine (MIO) agar test
 - Sulfide-indole-motility (SIM) agar test
6. Interpret quality control results for biochemical tests used for the identification of gram-negative bacteria.
7. Compare delayed lactose fermenters with nonlactose fermenters.
8. Discuss advantages of multitest systems.
9. Describe the significance of rapid reporting of bacterial identification.
10. Describe how established manual methods have been designed for the rapid identification of isolates.
11. Discuss the evaluation of identification systems.
12. Compare biochemical testing to molecular identification of microorganisms.

KEY TERMS

Asaccharolytic	Moeller decarboxylase base medium
β -galactosidase	Motility-indole-ornithine (MIO) agar
β -galactoside permease	Nitrate reduction test
Chromogenic substrate	Numeric codes
Citrate test	O/F basal medium
Deaminase	Ortho-nitrophenyl- β -D-galactopyranoside (ONPG)
Decarboxylase	Oxidase test
DNase	Oxidation
Fermentation	Phenotype
Fluorogenic substrate	Serotyping
Genotype	Sulfide-indole-motility (SIM) medium
Indole test	Triple sugar iron (TSI) agar
Kligler iron agar (KIA)	Urease test
Lysine iron agar (LIA)	Voges-Proskauer (VP) test
Matrix-assisted laser desorption/ionization–time-of-flight (MALDI-TOF) mass spectrometry	
Methyl red (MR) test	

Case in point

A 38-year-old male patient with a history of diabetes presented to the emergency department of a small rural hospital with fever and productive cough containing blood of several days' duration. The patient recently returned from Hainan Province, China, where he visited family members. A STAT sputum smear showed a moderate number of neutrophils (less than 10 squamous epithelial cells per high-power field) and a few gram-negative bacilli. A chest computed tomography revealed a patchy opacity in his left lung and multiple low-density lesions in his spleen. A sputum culture yielded growth of normal oral cavity microbiota and moderate growth of an oxidase-positive gram-negative bacillus. The Analytical Profile Index (API) multitest system gave a low percent identification as *Burkholderia pseudomallei*. The microbiology laboratory sent the specimen to a reference laboratory for confirmation.

Issues to consider

After reading the patient's case history, consider:

- What might be significant about this patient's travel to China?
- What are the advantages and disadvantages of multitest systems like the API to identify uncommon gram-negative bacilli?

Historically, the identification of bacteria has been based primarily on colony morphology characterization, microscopic morphology of organisms on Gram-stained smears, and biochemical testing. These methods are based on the **phenotype** of microorganisms, detecting observable or measurable characteristics. Biochemical testing was originally done in test tubes. Although this method was accurate, it required a lot of time to inoculate the multiple tubes of media and required lots of incubator space. Over time, biochemical tests were miniaturized and multitest systems were developed, resulting in

savings of time and space. Later, automated systems were developed. After inoculation, a computerized system records the test results, searches a database, and provides identification of the organism. Automated systems can provide preliminary identification and antimicrobial susceptibility patterns in a few hours. **Serotyping** or serogrouping, another example of phenotypic testing, is sometimes used with biochemical testing for identifying different strains of bacteria within a species. Serotyping uses antibodies to detect specific antigens located on the bacterial surface and is discussed in [Chapter 10](#).

Another phenotypic identification method is based on the analysis of fatty acids in microbial cell walls using gas liquid chromatography. Also, many modern microbiology laboratories now employ matrix-assisted laser desorption/ionization–time-of-flight (MALDI-TOF) mass spectrometry. This identification method is based on the characterization of microbial proteins. Some molecular biology assays are based on the **genotype** of the organism and are believed to be more accurate than examining the phenotype. Nucleic acid assays, based on nucleic acid sequences, available for many years now, are highly sensitive, specific, and rapid, providing accurate results in a few hours or less. Genotyping involves characterizing genes and, along with fatty acid analysis and MALDI-TOF mass spectrometry, will be discussed in [Chapter 11](#).

This chapter discusses the principles of conventional biochemical tests used commonly in the identification of gram-negative bacteria. [Box 9.1](#) lists some of these tests. Biochemical testing is becoming less important as clinical laboratories move to faster, more accurate molecular biology methodologies.

Carbohydrate utilization

Among bacteria, there is great diversity in the ability to use carbohydrates; however, determining lactose utilization is the carbohydrate determination test that is most important. Lactose degradation can be used to differentiate bacterial species able to ferment lactose (lactose fermenters) from species that are nonlactose fermenters. Lactose is a disaccharide consisting of glucose and galactose connected by a galactoside bond.

BOX 9.1 Traditional biochemical tests for identification of gram-negative bacteria

- Oxidase test to detect the presence of certain cytochrome C oxidases
- Triple sugar iron (TSI) agar or Kligler iron agar (KIA) to determine glucose and lactose, or sucrose, utilization (sucrose in TSI only) and hydrogen sulfide production
- Methyl red (MR) and Voges-Proskauer (VP) tests to determine end products of glucose fermentation
- Indole test to determine whether indole is formed from tryptophan by tryptophanase
- Urease test to determine hydrolysis of urea
- Simmons' citrate test to determine whether citrate can be used as the sole carbon source
- Carbohydrate fermentation
- Lysine iron agar (LIA) to determine lysine decarboxylase activity
- Sulfide-indole-motility (SIM) or motility-indole-ornithine (MIO) media

Two enzymes are necessary for a bacterium to take up lactose and to cleave it into monosaccharides: **β -galactoside permease** (lactose permease), which serves as a transport enzyme that facilitates entry of the lactose molecule across the bacterial plasma membrane, and **β -galactosidase**, the enzyme that hydrolyzes lactose into glucose and galactose. Lactose fermenters possess both β -galactoside permease and β -galactosidase, and nonlactose fermenters do not possess either enzyme. Some bacterial species lack β -galactoside permease but possess β -galactosidase. These bacterial species, termed *late* or *delayed lactose fermenters*, eventually can cleave the lactose molecule.

After lactose hydrolysis, glucose is available for metabolism primarily through the Embden-Meyerhof-Parnas (EMP) pathway, also referred to as *glycolysis*. Alternatively, bacteria can use the Entner-Doudoroff pathway (Fig. 9.1). See Fig. 1.10 in Chapter 1 for additional description of these pathways. Some bacterial species can only use glucose and are unable to attack the disaccharide lactose. Similarly, bacterial species incapable of fermenting glucose cannot use lactose.

Numerous other carbohydrates—monosaccharides, disaccharides, and polysaccharides—can be used by bacteria. Frequently these carbohydrates are ultimately converted into glucose for use in glycolysis. The polyhydric alcohols (which end in *-ol*), collectively called *sugars*, include adonitol, dulcitol, mannitol, and sorbitol. Some bacteria are not able to use any carbohydrates; instead, they use other organic molecules, such as amino acids, for energy and carbon sources. These organisms are called **asaccharolytic**.

Oxidation-fermentation tests

Bacteria can utilize carbohydrates by **oxidation** (aerobically), **fermentatively** (anaerobically), or both. Determining the oxidation-fermentation (O/F) pattern can be important in the identification of bacteria, particularly gram-negative bacilli. In particular, it is helpful in differentiating members of the order Enterobacterales, those bacteria that are glucose fermenters from the aerobic, nonfermenting pseudomonads.

Carbohydrate fermentation tests determine the ability of a microorganism to ferment a specific carbohydrate incorporated into a basal medium. During **fermentation**, glucose

enters the glycolysis pathway, resulting in the formation of pyruvic acid that can be broken down further into other acids. The end product of carbohydrate fermentation is acid or acid with gas. The pH indicators added to the medium detect acid formation. Some bacteria primarily produce a single acid, such as the streptococci, which are homolactic acid fermenters. Other bacteria produce several different acids and are referred to as *mixed acid fermenters*.

Oxidation also begins with glycolysis; however, the pyruvic acid formed from glycolysis is metabolized further to carbon dioxide (CO_2). Oxidation requires oxygen (aerobic respiration) or another inorganic molecule (anaerobic respiration), such as nitrate (NO_3^-), as a terminal electron acceptor. Greater acidity is produced during fermentation than during oxidation, but less energy is generated.

Characteristically, oxidizers and fastidious fermenters produce either weak or small amounts of acids from carbohydrates. In media that contain large amounts of peptones (1.0%), such as **triple sugar iron (TSI) agar**, whatever acids are produced are neutralized or masked by the alkaline reaction from peptone utilization. To detect small amounts of acids produced, whether fermentatively or oxidatively, Hugh and Leifson developed an **O/F basal medium** (OFBM) that contains the same concentration of carbohydrates (1%) found in TSI medium but a lower concentration of peptones (0.2%). The pH indicator is bromothymol blue. Uninoculated medium is green; in an acid environment, the indicator is yellow; and in an alkaline environment, it is blue (Fig. 9.2).

Table 9.1 shows the differences in reactions among different groups of organisms. The same medium is used for both oxidative and fermentative tests. When O/F tests are performed, two tubes of Hugh-Leifson OFBM are inoculated: one tube is overlaid with sterile mineral oil to create an anaerobic environment (closed), and the other is left aerobic (open), without mineral oil overlay. When acid is produced in both tubes, the isolate is likely a fermenter. Fermentation can

Embden-Meyerhof-Parnas Pathway Entner-Doudoroff Pathway

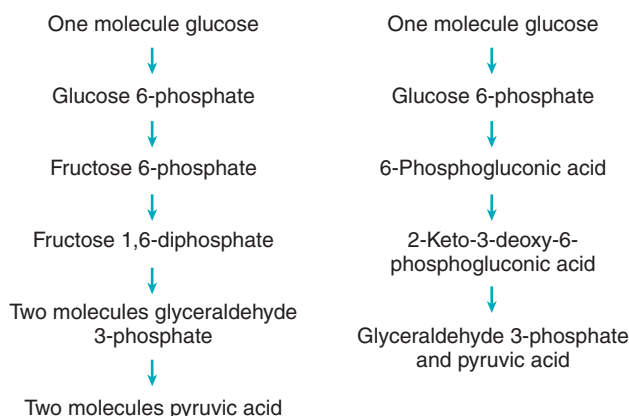


Fig. 9.1 Two pathways for glucose degradation.

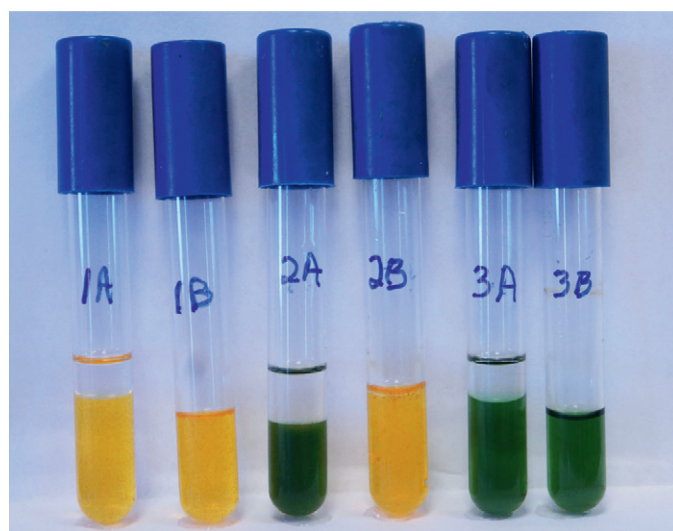


Fig. 9.2 Reactions in oxidative fermentative media. The pair of tubes on the *left* is representative of a fermenter: open and oil-overlaid tubes are positive for acid. The pair of tubes in the *center* is representative of an oxidizer/nonfermenter: acid is produced only in the open tube. The pair of tubes on the *right* is representative of a nonoxidizer/nonfermenter: the oil-overlaid tube is unchanged, and the open tube is alkaline from peptone utilization.

Table 9.1 Biochemical reactions characteristic of gram-negative bacilli on TSI, KIA, and OFBM Media

	TSI agar	KIA	Hugh-leifson OFBM
Carbohydrates (concentration)	Glucose (0.1%)	Glucose (0.1%)	Glucose or other carbohydrate being tested (1%)
	Lactose (1%)	Lactose (1%)	
	Sucrose (1%)		
Peptone	2%	2%	0.2%
Fermenter	Acid butt	Acid butt	Open tube: acid
	Acid or alkaline slant	Acid or alkaline slant	Sealed tube: acid
Nonfermenter			
	Oxidizer		
	Alkaline butt	Alkaline butt	Open tube: acid
	Alkaline slant	Alkaline slant	Sealed tube: no acid
Nonoxidizer (asaccharolytic)	Alkaline butt	Alkaline butt	Open tube: no acid
	Alkaline slant	Alkaline slant	Sealed tube: no acid

KIA, Kligler iron agar; OFBM, oxidation-fermentation basal medium; TSI, triple sugar iron.

occur aerobically or anaerobically, if the bacterium is unable to oxidize the carbohydrate. This situation occurs with many streptococci, which derive all energy from fermentation, even in the presence of oxygen. The presence of acid in the closed tube only indicates that the organism is a fermenter and possible obligate anaerobe, whereas the presence of acid in the open tube only indicates an oxidizer. The open tube may or may not show acidity. No acid production in the open tube may indicate that the organism is a nonoxidizer; many nonoxidizers produce an alkaline reaction from peptone utilization.

Triple sugar iron agar

TSI agar and **Kligler iron agar (KIA)** are useful in the presumptive identification of gram-negative enteric bacteria, particularly in screening for intestinal pathogens. The formulas for TSI agar and KIA are identical except that TSI agar contains sucrose in addition to glucose and lactose. Lactose is present in a concentration 10 times that of glucose (1% lactose and 0.1% glucose). In TSI agar, sucrose is also present in a 1% concentration. Ferrous sulfate and sodium thiosulfate are added to detect the production of hydrogen sulfide gas (H_2S). Phenol red is used as the pH indicator, which is yellow below a pH of 6.8. Uninoculated medium is red because the pH is buffered at 7.4. Both TSI agar and KIA are useful in detecting the ability of bacteria to ferment carbohydrates (glucose and lactose in KIA and glucose, lactose, or sucrose in TSI agar); to produce gas from the fermentation of sugars; and to detect the production of H_2S .

Both TSI agar and KIA are poured on a slant. The slant portion is aerobic; the butt, or deep portion, is anaerobic. To

inoculate TSI agar or KIA, the laboratory scientist should pick a well-isolated colony with an inoculating needle and stab the butt almost all the way to the bottom of the tube. The laboratory scientist removes the needle and then uses a back-and-forth motion across the surface of the slant. The cap is replaced loosely to allow oxygen to enter the tube, and the medium is incubated in a non- CO_2 incubator for 18 to 24 hours. The reaction patterns are written with the slant results first, followed by the butt reaction, separated by a slash (slant reaction/butt reaction). It is important that the reactions be read within 18 to 24 hours; otherwise, erroneous results are possible.

Reactions on triple sugar iron agar or kligler iron agar

1. *No fermentation: alkaline slant/alkaline butt (K/K) or alkaline slant/no change (K/NC).* Although unable to ferment either lactose or glucose, these organisms can degrade the peptones present in the medium aerobically or anaerobically, resulting in the production of alkaline by-products in the slant or deep, respectively, changing the indicator to a deep red color. These reactions are typical of organisms that are not members of the order Enterobacterales.
2. *Glucose fermentation only, no lactose (or sucrose in TSI) fermentation: alkaline slant/acid butt (K/A).* TSI agar and KIA contain glucose in a 0.1% concentration. The acid produced from this concentration of glucose is enough to change the indicator to yellow initially throughout the medium. However, after about 12 hours, the glucose is consumed, and bacteria on the slant utilize the peptones aerobically, producing an alkaline reaction, which changes the indicator to a deep red color. Fermentation of glucose (anaerobic) in the butt produces larger amounts of acid, overcoming the alkaline effects of peptone degradation; therefore the butt remains acidic (yellow). Reading the results after less than 12 hours of incubation (acid/acid) gives the false appearance of an organism capable of fermenting glucose and lactose (or sucrose in the case of TSI agar). For this reason, TSI agar or KIA must be incubated for 18 to 24 hours.
3. *Lactose and/or sucrose fermentation: acid/acid (A/A).* Glucose fermenters attack the simple sugar glucose first and then lactose or sucrose. The acid production from the fermentation of the additional sugar is sufficient to keep both the slant and the butt acidic (yellow) when examined at the end of 18 to 24 hours of incubation. If the medium is incubated beyond 24 hours, however, it is possible that the lactose or sucrose could be consumed, and an alkaline slant could be formed from peptone utilization. It is important that the TSI agar and KIA tests are not read after 24 hours of incubation.
4. *H_2S production: alkaline slant/acid butt, H_2S in butt (K/A, H_2S) or acid slant/acid butt, H_2S in butt (A/A H_2S).* H_2S production is a two-step process. In the first step, H_2S is formed from sodium thiosulfate. Because H_2S is a colorless gas, the indicator, ferrous sulfate, is necessary to visually detect its production. In some cases, the butt of the tube is completely black, obscuring the yellow color from carbohydrate fermentation. Because H_2S production requires an acid environment, even if the yellow color cannot be seen, it is safe to assume glucose fermentation.

- a. Bacterium (acid environment) + sodium thiosulfate → H₂S gas
- b. H₂S + ferric ions → ferrous sulfide (black precipitate)
5. Gas production (aerogenic) or no gas production (nonaerogenic). The production of gas results in the formation of bubbles or splitting of the medium in the butt or complete displacement of the medium from the bottom of the tube. Fig. 9.3 illustrates the reactions on TSI agar.

Ortho-nitrophenyl-β-D-galactopyranoside test

Organisms that are delayed lactose fermenters appear initially as nonfermenting colonies on primary isolation media. When placed on TSI agar or KIA slants, these species produce similar results after 18 to 24 hours of incubation. The **ortho-nitrophenyl-β-D-galactopyranoside** (ONPG) and the *p*-nitrophenyl-β-D-galactopyranoside (PNPG) tests determine whether the organism is a delayed lactose fermenter (one that lacks the enzyme β-galactoside permease but possesses β-galactosidase) or a true nonlactose fermenter. Structurally, ONPG is similar to lactose, but ONPG is more readily transported through the bacterial plasma membrane and does not require β-galactoside permease. β-Galactosidase hydrolyzes ONPG, a colorless compound, into galactose and *o*-nitrophenol, a yellow compound. ONPG remains colorless if the organism is a nonlactose fermenter (Fig. 9.4).

The test can be performed by making a heavy suspension of bacteria in sterile saline and adding commercially prepared ONPG disks or tablets. The suspension is incubated at 35° C, and positive results can generally be seen within 6 hours. β-Galactosidase is an inducible enzyme. The gene is expressed only if lactose or a similar substrate is present; therefore bacteria should be taken from lactose-containing media for testing. Bacteria producing a yellow pigment should not be tested because of the risk of a false-positive result.

Glucose metabolism and its metabolic products

Glucose metabolized via the Embden-Meyerhof pathway produces several intermediate by-products, including pyruvic acid. Further degradation of pyruvic acid can produce mixed acids as end products. However, enterics take two separate pathways: the mixed acid fermentation pathway or the butylene glycol pathway. The **methyl red (MR) test** and the **Voges-Proskauer (VP) test** detect the end products of glucose fermentation. Each test detects products from a different pathway.

Methyl red test

Bacteria are incubated in a broth medium containing glucose. Most commercial methyl red–Voges-Proskauer (MRVP) media are a modification of Clark and Lubs medium. The broth should be incubated 3 to 5 days at 35° C. After incubation, approximately one half of the broth is transferred to a clean, dry tube for the VP test. If glucose is metabolized by the mixed acid fermentation pathway, stable acid end products are produced, which results in a low pH. A red color develops after



Fig. 9.3 Triple sugar iron agar reactions. Left to right, A/A gas; A/A H₂S; K/A; K/A H₂S; K/K.



Fig. 9.4 Ortho-nitrophenyl-β-D-galactopyranoside (ONPG) test. (Courtesy American Society for Clinical Laboratory Science, Education and Research Fund, Inc., 1982.)

addition of the pH indicator MR (Fig. 9.5). MR-negative cultures remain yellow after addition of the pH indicator (pH 6.0).

Glucose → Pyruvic acid → Mixed acid fermentation (pH 4.4)



Red color with methyl red indicator

Voges-Proskauer test

In some bacteria, acids formed during fermentation can be metabolized further to 2,3-butanediol through the intermediate acetoin. After incubation, α-naphthol is added first as a catalyst or color intensifier. Next, 40% potassium hydroxide (KOH) or sodium hydroxide (NaOH) is added, and the tube is gently shaken to increase oxygenation. Under these

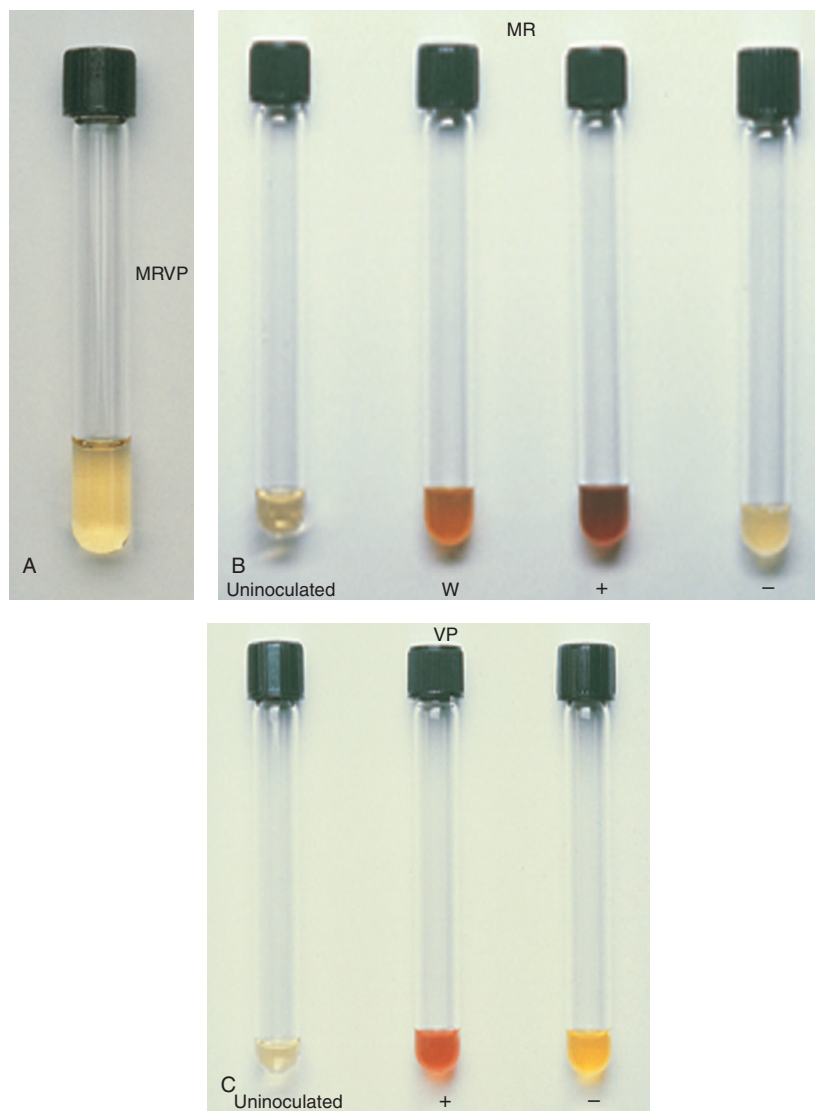
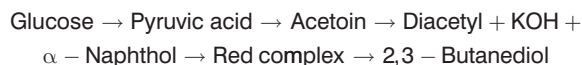


Fig. 9.5 **A**, Methyl red–Voges-Proskauer (MRVP) test is inoculated and incubated overnight. It is then split equally into two parts: one part for the methyl red (MR) test, the other for the Voges-Proskauer (VP) test. **B**, MR test. **C**, VP test. W, Weak positive. (Courtesy American Society for Clinical Laboratory Science, Education and Research Fund, Inc., 1982.)

conditions, acetoin is oxidized to diacetyl. Diacetyl in the presence of KOH and α -naphthol forms a red complex. The pH remains relatively neutral. Fig. 9.5 illustrates the MRVP test. Bacteria tend to be either MR or VP positive, but not both. Some bacteria are negative for both tests.



Amino acid utilization

Decarboxylase and dihydrolase tests

Many bacteria can use amino acids as energy and carbon sources. **Decarboxylase** tests determine whether the bacterial species possess enzymes capable of decarboxylating (removing the carboxyl group, COOH) specific amino acids in the test medium. Lysine and ornithine are two amino acids

commonly used to test for decarboxylase activity. The products of decarboxylation are amine or diamine molecules and CO₂, with resulting alkalinity. Degradation of the amino acids and their specific end products are shown in the following reaction:

Degradation of amino acids and their specific end products

Lysine (amino acid) $\rightarrow$ Lysine decarboxylase $\rightarrow$

Cadaverine (amine) + CO₂

Ornithine $\rightarrow$ Ornithine decarboxylase $\rightarrow$ Putrescine

Arginine $\rightarrow$ Arginine dihydrolase $\rightarrow$ Citrulline $\rightarrow$

Ornithine $\rightarrow$ Putrescine

Lysine is decarboxylated by the enzyme lysine decarboxylase to cadaverine, a diamine, and CO₂. Ornithine is cleaved by ornithine decarboxylase to putrescine, a diamine, and CO₂. Both cadaverine and putrescine are stable in anaerobic conditions. Arginine can be decarboxylated in a two-step process. In the first step, arginine undergoes decarboxylation by arginine decarboxylase to form agmatine and CO₂. Then agmatine

is metabolized further to putrescine and urea. If the bacteria also produce urease, the urea is degraded to ammonia (NH_3) and CO_2 . Arginine also can be degraded by arginine dihydrolase forming citrulline, ammonia, and inorganic phosphate. In the next step, citrulline undergoes phosphorolytic cleavage to yield ornithine. If the bacteria also possess ornithine decarboxylase, ornithine is converted to putrescine.

The test to detect decarboxylation uses **Moeller decarboxylase base medium**. The broth contains glucose; peptones; two pH indicators, bromocresol purple and cresol red; and the specific amino acid at a concentration of 1%. The medium has an initial pH of 6.0. Having glucose in the medium is important because decarboxylases are inducible enzymes produced in an acid pH. The uninoculated medium is purple; metabolism of the small amount of glucose decreases the pH to about 5.5, turning the medium yellow. For decarboxylation to take place, two conditions must be met: an acid pH and an anaerobic environment. A control tube containing only the base medium without the amino acid is inoculated to determine the viability of the organism. The control tube also determines whether sufficient acid is produced. Both tubes are inoculated with the test organism; overlaid with a layer of sterile mineral oil, which creates anaerobic conditions; and then incubated at 35° C.

During the first few hours of incubation, organisms attack the glucose first, changing the pH to acid. If the organism produces the specific decarboxylase and the amino acid in the medium is attacked, release of the amine products causes an alkaline pH shift, resulting in a purple (positive result) color in the medium. If the organism does not possess the specific decarboxylase, the medium remains yellow (negative result). The control tube should remain yellow. Results can usually be recorded in 24 hours; however, bacteria with weak decarboxylase activity may take 4 days to be positive. Modifications of the decarboxylase test to detect other biochemical reactions are also used. Examples of these include the MIO and LIA tests (Fig. 9.6).



Fig. 9.6 Lysine iron agar reactions. *Left to right*, K/K (positive decarboxylation without H₂S), K/A H₂S (negative decarboxylation with H₂S), K/K H₂S (positive decarboxylation with H₂S), R/Y (negative decarboxylation, positive deamination without H₂S).

Deaminase test

Amino acids can be metabolized by **deaminases** that remove an amine (NH_2) group. The phenylalanine deaminase (PAD) test determines whether an organism possesses the enzyme that deaminates phenylalanine to phenylpyruvic acid. The test medium is an agar slant containing a 0.2% concentration of phenylalanine. The surface of the slant is inoculated with a bacterial colony. After incubation, addition of a 10% ferric chloride (FeCl_3) reagent results in a green color if phenylpyruvic acid is present. This test is helpful in initial differentiation of *Proteus*, *Morganella*, and *Providencia* organisms, which are positive, from the rest of the Enterobacterales (Fig. 9.7).

Deamination of Phenylalanine

Phenylalanine → Phenylalanine deaminase →
Phenylpyruvic acid + (FeCl_3) green

Miscellaneous tests

Citrate utilization

The **citrate test** determines whether an organism can use sodium citrate as a sole carbon source. Simmons' citrate medium is frequently used for this test. In addition to citrate, the test medium contains ammonium salts as the sole nitrogen source. Bacteria able to use citrate will use the ammonium salts, releasing ammonia. The alkaline pH that results from ammonium salts utilization changes the pH indicator (bromothymol blue) in the medium from green to blue. It is important to use a light inoculum because dead organisms can be a source of carbon, producing a false-positive reaction (Fig. 9.8). Christensen's citrate medium is an alternative test medium. This medium incorporates phenol red (as the pH indicator) and organic nitrogen. At an alkaline pH, the indicator turns from yellow to pink.

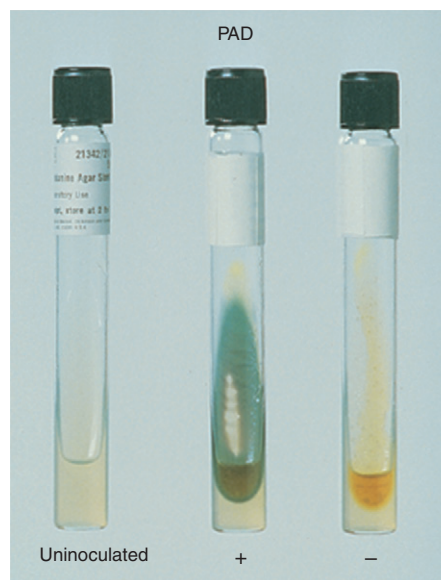


Fig. 9.7 Phenylalanine deaminase (PAD) test. (Courtesy American Society for Clinical Laboratory Science, Education and Research Fund, Inc., 1982.)



Fig. 9.8 Citrate utilization test. *Left*, Uninoculated. *Center*, Positive result. *Right*, Negative result.

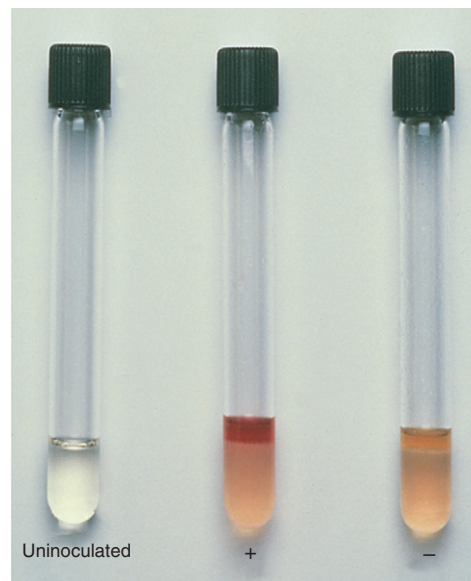


Fig. 9.9 Indole broth. (Courtesy American Society for Clinical Laboratory Science, Education and Research Fund, Inc., 1982.)

Deoxyribonuclease (DNase) production

Deoxyribonucleic acid (DNA) is a polynucleotide composed of repeating purine and pyrimidine mononucleotide monomeric units. Most bacterial deoxyribonucleases (**DNases**) are endonucleases cleaving internal phosphodiester bonds, resulting in smaller subunits. Extracellular DNase can be produced by numerous bacteria, such as *Staphylococcus aureus* and *Serratia marcescens*. DNase test medium usually contains 0.2% DNA. A heavy inoculum of bacteria is streaked onto the surface of the medium in a straight line; several organisms can be tested at once. The plate is incubated at 35° C for 18 to 24 hours, and then 1 N HCl is added to the surface of the plate. Unhydrolyzed DNA is insoluble in HCl and forms a precipitate. Oligonucleotides formed from the action of DNase dissolve in the acid, forming a clear zone (halo) around the inoculum, a positive result. An alternate formulation incorporates methyl green into the medium. Methyl green complexes with DNA forming a green color. If the DNA is broken down by bacterial DNases, DNA can no longer bind methyl green, and the green color fades. A clear zone around the bacteria indicates a positive result.

Indole production

Indole is one of the degradation products of the amino acid tryptophan. Organisms that possess the enzyme tryptophanase are capable of deaminating tryptophan with the formation of the intermediate degradation products of indole, pyruvic acid, and ammonia. Bacteria are inoculated into tryptophan or peptone broth. Most commercial peptone broth contains enough tryptophan for a positive reaction; tryptophan can be added to a final concentration of 1%. After inoculation, the broth should be incubated at 35° C for 48 hours. After incubation, one of two methods can be used to detect indole. In the Ehrlich **indole test**, the indole is extracted from the broth culture by the addition of 1 mL of xylene. After the xylene is added, the tube is shaken well. After waiting a few

minutes for the xylene to rise to the top, 0.5 mL of Ehrlich's reagent, containing *p*-dimethylaminobenzaldehyde (PDAB), is added. If indole is present, a red color develops after the addition of PDAB (Fig. 9.9). Alternatively, Kovac's reagent, which also contains PDAB, can be used. This method does not use a xylene extraction. Approximately five drops of Kovac's reagent are added directly to the broth culture. The tube is shaken, and if indole is present, a red color develops. The Ehrlich method is more sensitive than Kovac's reagent and is preferred with nonfermentative bacteria. If indole-nitrate (trypticase nitrate) medium is used, the indole test can be performed from the same broth culture as a nitrate test. Before adding any reagents, the broth is divided in half, one aliquot for the indole test and the other for the nitrate test. A rapid indole test is available. Isolated bacterial colonies are smeared onto filter paper that has been moistened with *p*-dimethylaminocinnamaldehyde. The formation of a blue-green color within 2 minutes is a positive reaction indicating the presence of indole in the bacterial colony.

Lysine iron agar slant

The **lysine iron agar (LIA)** test is a tubed agar slant containing the amino acid lysine, glucose, ferric ammonium citrate, and sodium thiosulfate. The pH indicator is bromocresol purple. LIA is used primarily to determine whether bacteria decarboxylate or deaminate lysine (see Fig. 9.6). H₂S production is also detected in this medium. LIA is inoculated in the same manner as a TSI agar slant. LIA is most useful in conjunction with TSI in screening stool specimens for the presence of enteric pathogens, differentiating *Salmonella* spp. (lysine-positive) from *Citrobacter* spp. (lysine-negative). Decarboxylation only occurs anaerobically; the presence of a dark purple butt is positive for lysine decarboxylation. The production of H₂S can mask the purple color in the butt of the tube. Because H₂S production in LIA occurs only in an alkaline environment, a black precipitate indicating H₂S is also a positive result for decarboxylation. LIA is also useful in differentiating *Proteus*,

Morganella, and *Providencia* spp. from most other members of the order Enterobacterales. This group of enterics deaminates (attacks the NH_2 group instead of the carboxyl group) amino acids. In the LIA slant, deamination of lysine turns the original light purple color slant to a plum or reddish-purple color; the butt turns yellow because of glucose fermentation.

Case check 9.1

In the Case in Point at the beginning of the chapter, the patient's history and laboratory culture results implied an infection caused by *B. pseudomallei*. This bacterium causes the disease melioidosis, which is uncommon in the United States. It is most prevalent in tropical regions such as Southeast Asia. Nonfermentative gram-negative bacilli tend to be more difficult to identify biochemically because they have fewer positive reactions. In addition, uncommon isolates tend to be more difficult to identify in commercial identification systems partly because of an insufficient number of known samples analyzed for the system's database.

Motility

Motility can be determined by microscopic examination of bacteria or by observing growth in a semi-solid medium. Motility test media have agar concentrations of 0.4% or less to allow for the free spread of microorganisms. A single stab is made into the center of the tubed medium. Best results are obtained if the stab is made as straight as possible. After incubation, movement away from the stab line or a hazy appearance throughout the medium indicates a motile organism. Incubation temperature is important. Some bacteria are motile only at room temperature, but this temperature may not be optimal for growth. It is suggested that two motility tubes be inoculated, one incubated at room temperature and the other at 35° C. Comparing inoculated with uninoculated tubes may help in interpreting results.

Motility-indole-ornithine agar

Motility-indole-ornithine (MIO) agar is a semi-solid agar medium used to detect motility and indole and ornithine decarboxylase production. MIO is useful in differentiating *Klebsiella* spp. from *Enterobacter* and *Serratia* spp. The medium is inoculated by making a straight stab down the center of the medium with an inoculating needle. Motility is shown by a clouding of the medium or spreading growth from the line of inoculation. Ornithine decarboxylation is indicated by a purple color throughout the medium. Because MIO is a semi-solid medium, it does not have to be overlaid with mineral oil to provide anaerobic conditions. Indole production is detected by the addition of Kovac's reagent; a pink to red color is formed in the reagent area if the test result is positive. Ornithine decarboxylase and motility should be read first before the addition of Kovac's reagent.

Nitrate and nitrite reduction

The **nitrate reduction test** determines whether an organism has the ability to reduce nitrate to nitrite and reduce nitrite further to nitrogen gas (N_2). The organism is inoculated into

a nutrient broth containing a nitrogen source. After 24 hours of incubation, *N,N*-dimethyl- α -naphthylamine and sulfanilic acid are added. A red color indicates the presence of nitrite.

Nitrate reduction test reaction

Nutrient broth with 0.1% potassium nitrate $\rightarrow$

Nitrate reductase $\rightarrow$ Nitrite $\rightarrow$ Sulfanilic acid +

N,N – Dimethyl – α – naphthylamine $\rightarrow$ Diazo red dye

If no color develops, this may indicate that nitrate has not been reduced or that nitrate has been reduced further to N_2 , nitric oxide (NO), or nitrous oxide (N_2O), which the reagents will not detect. Adding a small amount of zinc dust will help determine whether the test has produced a true-negative result or whether the lack of color production was caused by reduction beyond nitrite. Zinc dust reduces nitrate to nitrite. Therefore development of a red color after the addition of zinc confirms a true-negative test result. Alternatively, a small glass tube, called a *Durham tube*, can be inserted into the broth upside down when the medium is aliquoted into test tubes. During incubation, if nitrogen gas is produced, it will be trapped in the inverted Durham tube.

Oxidase

The **oxidase test** determines the presence of the cytochrome oxidase system that oxidizes reduced cytochrome with molecular oxygen. The oxidase test is helpful in differentiating between Enterobacterales, which are oxidase-negative (except for *Plesiomonas*), and the pseudomonads, which are oxidase-positive. The oxidase test is also useful in identifying *Neisseria* spp., which are oxidase positive. A modified oxidase test is used to distinguish *Staphylococcus* from *Micrococcus*. Several methods for performing an oxidase test are available. Kovac's oxidase test uses a 0.5% or 1% aqueous solution of tetramethyl-*p*-phenylenediamine dihydrochloride. A drop of the reagent is added to filter paper, and a wooden applicator stick is used to rub a colony onto the moistened filter paper. The development of a lavender color within 10 to 15 seconds is a positive reaction. *p*-Aminodimethylaniline oxalate is less sensitive than tetramethyl-*p*-phenylenediamine dihydrochloride, but it is cheaper and more stable. Commercial forms of oxidase reagent are available in glass ampules and on filter paper disks. An oxidase test should not be performed on bacteria grown on plates with dyes, like eosin-methylene blue, because this can result in false-positive results.

Sulfide-indole-motility agar

Sulfide-indole-motility (SIM) medium is a semi-solid agar helpful in differentiating gram-negative bacteria in the order Enterobacterales. An inoculating needle is used to make a straight stab down the center of the medium. Cloudiness spreading from the inoculation line is positive for motility. The production of H_2S is indicated by a black precipitate, and a pink to red color after the addition of Kovac's reagent is positive for indole.

Urease

The **urease test** determines whether a microorganism can hydrolyze urea, releasing enough ammonia to produce a

color change by a pH indicator. Urease hydrolyzes urea to form ammonia, water, and CO₂. Different formulations of urea agar are available, but Christensen's urea agar is generally preferred. The surface of the agar slant is inoculated but not stabbed. The medium contains phenol red as the pH indicator. The resulting alkaline pH from hydrolysis of urea is indicated by a bright pink color (Fig. 9.10).

Case check 9.2

Identification confirmation for uncommonly isolated nonfermentative gram-negative bacilli is often based on genomic nucleic acid sequencing or MALDI-TOF mass spectrometry. The rural hospital in this case requested confirmation from a regional medical center. The identification was confirmed by genomic sequencing.

Manual multitest systems

Principles of identification

Microbial identification methods fall into one of five categories or a combination thereof: (1) pH-based reactions, (2) enzyme-based reactions, (3) utilization of carbon sources, (4) visual detection of bacterial growth, or (5) molecular assays based on the characterization of molecules including volatile or nonvolatile fatty acids, nucleic acids, and proteins. Immunoassays, discussed in Chapter 10, rely on antibodies to detect microbial antigens. Most of the assays require microbial growth, but detection of nucleic acids does not (Table 9.2). The molecular assays are discussed in Chapter 11.

Identification of bacteria and yeasts can be facilitated by using automated or packaged kit systems by which organisms are identified with computer-assisted databases or computer-derived **numeric codes**. These numeric codes are generated based on the metabolic profiles of each organism.



Fig. 9.10 Urease test. Left, Negative result. Right, Positive result.

Each metabolic reaction, or phenotype, is recorded as plus (+) for positive reactions and minus (–) for negative reactions. These plus-minus sequences are cataloged as binary numbers and stored in a computer database. Binary codes are computer-converted to code profile numbers that represent the identifying phenotype of specific organisms. After metabolic profiles have been converted to numbers, a percent probability of correct identification is assigned based on the comparison of the unknown profile with known profiles within the database. As more organisms are included in the database, the genus and species designations and probabilities become more precise. With the frequent name changes that occur, it is sometimes difficult to maintain the current taxonomy in a database. All commercial suppliers of multicomponent biochemical test systems provide users with one or more of the following: a computer with profile number database, a computer-derived code book, access to a website, or access to a telephone inquiry center to facilitate matching profile numbers with species.

Analytical profile index

The Analytical Profile Index (API; bioMérieux, Durham, NC) was released in 1970. The system for identification of gram-negative fermentative bacteria (the order Enterobacterales) is called the *API 20E*. This system has a series of 20 cupules attached to a plastic strip. Inside the cupules are lyophilized, pH-based substrates. A bacterial suspension made in saline is used to rehydrate the reagents in the cupules. The principles of the tests are the same or similar to the principles of tests performed in test tubes. Some of the cupules, such as those for amino acid deaminases and dehydrolase, require a mineral

Table 9.2 Methodologies for microbial identification		
System	Growth needed?	Comment
pH-based	Yes	Carbohydrate and amino acid utilization (e.g., API)
Enzyme profile	No	Rapid results (2 to 4 hours) based on the presence of preformed enzymes (e.g., MicroScan rapid panels)
Carbon source utilization	Yes	Color change based on transfer of electrons to colorless indicator, producing a colored compound (e.g., Biolog)
Volatile or nonvolatile compound detection	Yes	Fatty acids detected by chromatographic assays (e.g., MIDI)
Visual detection of growth	Yes	Growth determined by presence of turbidity (e.g., MicroScan)
MALDI-TOF mass spectrometry	Yes	Protein patterns determined by mass spectrophotometry (e.g., Bruker)
Target DNA	No	Sequence target DNA and compare to public database or nucleic amplification and probe detection (e.g., Gen-Probe)
API, Analytical Profile Index; MALDI-TOF, matrix-assisted laser desorption/ionization–time-of-flight.		

oil overlay to achieve anaerobiosis. The strip is incubated 18 to 24 hours at 35° C, and reagents are added to some of the cupules. The last test used to determine the profile number is the oxidase test, which is not part of the API strip. Results are recorded, and a 7-digit code profile number is determined.

After determining the code profile number, a database provided by the manufacturer is consulted, providing the most probable identification. If the identification is in question, nitrate reduction and motility are supplemental tests that can be used to obtain an additional digit for the profile number. The API system has been a standard product in many clinical microbiology laboratories and has remained unchanged. Accuracy for commonly isolated members of the order Enterobacterales, such as *Escherichia coli*, *Klebsiella pneumoniae*, and *Proteus mirabilis*, has been reported to be 87.7% at 24 hours of incubation. For less commonly isolated Enterobacterales, the accuracy is 78.7% at 24 hours. bioMérieux markets many multitest API systems, including systems for gram-positive cocci, nonfermentive gram-negative bacilli (20NE), yeasts (API 20C), and a rapid system for identification of Enterobacterales in 4 hours (RapID 20E).

Other multitest systems include Crystal E/NF (BD Diagnostic Systems, Sparks, MD), RapID NF Plus (Thermo Fisher Scientific, Waltham, MA), Microbact Biochemical Identification (Thermo Fisher Scientific), Enterotube II (BD Diagnostic Systems), and Micro-ID (Thermo Fisher Scientific). Biolog (Hayward, CA) markets the Gen III MicroLog M manually read system.

Rapid and automated identification systems

Until the late 1970s, microbiologists relied on the growth and isolation of bacteria in broth and agar media. Once bacteria are cultured in vitro, their biochemical or metabolic characteristics can be used for identification. Isolation of the infectious agent from clinical samples typically requires 24 to 48 hours. Identification protocols often require another 24 hours of incubation in the presence of specific biochemical substrates. Rapid diagnosis of infectious disease remains a challenge for the clinical microbiology laboratory. The rapid and accurate reporting of results should positively affect patient outcomes by quick initiation of appropriate antimicrobial therapy. This allows the clinical microbiology laboratory to have a proactive, rather than retrospective, effect on patient management.

Rapid reporting also becomes increasingly important considering today's diagnostic challenges. Newly emerging pathogens, recognition of old pathogens in different clinical settings, world travel, increases in healthcare-associated infections, and the prevalence of multidrug-resistant organisms all contribute to the need for the design and development of new identification capabilities. Some of the major concepts and applications of rapid methods and automation currently available for the identification of clinically significant microorganisms are listed in the following sections. Although not exhaustive, the principles of most rapid identification technologies found in the clinical microbiology laboratory have been included. Other rapid identification methods are discussed in [Chapters 10](#) and [11](#). Specific identification methods are discussed in greater detail in chapters in association with specific organism descriptions.

The term *rapid*

Direct microscopic examination of body fluids provides results within 15 to 30 minutes; these results are often valuable in patient management. For example, Gram stain results from a cerebrospinal fluid specimen along with blood and spinal fluid chemistry (glucose and protein) and hematology (complete blood count and differential) may be critical in establishing the cause of infectious meningitis. However, direct microscopy generally has a low sensitivity. [Chapter 7](#) discusses the microscopic examination of clinical samples.

For decades, microbiologists relied on the ability of an organism to ferment sugars, degrade amino acids, and produce unique end products for identification purposes. Diagnosis of an infectious disease has been a complex, laborious, and frequently slow process. In the late 1950s and 1960s, traditional biochemical tests became miniaturized. Smaller test tubes and molded plastic vessels were introduced. These changes made testing more convenient but did not improve turnaround times in reporting results. In the 1970s, microbiologists began to rely on computerized databases so numerous results could be considered simultaneously, and the most statistically probable result could be regarded as the identification of the unknown organism. This improved the reliability of results but still did not improve reporting turnaround times. In the mid- to late 1970s, semiautomated instruments for identification and susceptibility testing gradually appeared. In many laboratory settings, these instruments shortened turnaround times, yielded greater accuracy, and improved productivity.

The phrase *rapid method* encompasses a wide variety of procedures and techniques and has been loosely applied to any procedure affording results faster than the conventional method. Rapid methods exist for microscopy, biochemical identification, antigen detection, and antibody detection. *Rapid* is a relative term used to describe time, and it depends on the procedures being compared. For example, a 3-hour enzyme immunoassay method to detect *Clostridioides difficile* toxin is rapid compared with a 48-hour cell culture cytotoxicity assay. The fluorescence quenching-based oxygen sensor detection of *Mycobacterium tuberculosis* within 2 weeks with the BACTEC 9000 MB (BD Diagnostic Systems) is rapid compared with the 6 weeks required to grow the organism on agar slants.

Microscopic procedures using common stains and fluorescent-labeled antibody to detect specific organisms are rapid methods; so are some conventional procedures used for initial differentiation or presumptive identification of certain groups of organisms. These procedures have been modified to provide immediate results that may lead to presumptive identification. Rapid identification of clinical isolates by biochemical reactions often involves commercially packaged identification kits or fully automated instruments. These manufactured kits are usually miniaturized test systems that employ chromogenic or fluorogenic substrates to assess preformed enzymes. **Chromogenic substrates** are colorless; when cleaved by a microbial enzyme, a colored compound is produced. **Fluorogenic substrates** are nonfluorescent until cleaved by microbial enzymes. Reaction endpoints may be reached after 2 to 6 hours of incubation, although some may require overnight incubation. The capabilities of these kits and systems vary widely. Certain systems may still require manual reading

by the laboratory scientist, whereas others may use mechanized reading through spectrophotometry, numeric coding, and computerized databases. Some of these instruments also incubate, read, and interpret the enzymatic test results.

Rapid biochemical tests performed on isolated colonies

Table 9.3 summarizes the principles, modes of action, and applications of certain established manual procedures for quick presumptive differentiation among groups of organisms or presumptive identification to bacterial species. Often more than one test must be performed for a presumptive identification. These tests may also provide direction about additional tests needed for definitive identification. Further discussion of these and other rapid biochemical methods is included in subsequent chapters as they apply to identification of specific organisms.

Identification systems relying on carbohydrate utilization or chromogenic substrates

Rapid tests for detection of end products resulting from carbohydrate metabolism or enzymatic tests using chromogenic

substrates produce reaction endpoints in minutes to hours. Plastic cupules, reaction chambers, or filter paper strips contain desiccated or dehydrated reagents or substrates. In general, a suspension of bacterial cells or a loopful of an isolated colony is added to the system or rubbed off to a reaction area. A positive reaction is measured by enzymatic activity and color change.

Multitest kits using conventional carbohydrate metabolism take advantage of one test inoculum distributed to multiple reaction sites to yield more than one result. To obtain more rapid results, conventional methods have been modified by decreasing the test substrate medium volume and increasing the concentration of bacteria in the inoculum. Methods based on enzyme substrates have certain advantages over conventional methods. Because enzymatic methods involve preformed enzymes, they do not require multiplication of the organism (growth independent). Endpoints are reached in minutes to a few hours. The tests are very sensitive for the presence of the enzyme, although not always specific for genus and species identification; however, sensitivity depends on the concentration and stability of the substrate, the enzyme, and the age of the inoculum. Several rapid modifications of conventional methods for bacterial and yeast identification are listed in Table 9.4. Thermo Fisher Scientific markets a series of RapID panels that can provide identification in about 4 hours (Fig. 9.11).

Table 9.3 Rapid biochemical tests performed on isolated colonies

Test	Bacterial enzyme	Mode of action	Applications
Spot indole	Tryptophanase	Organism from blood agar or any tryptophan-containing medium is placed on a swab, and reagent is added; hydrolysis of tryptophan to indole is indicated by production of blue to blue-green color on addition of DMAC	Positive reaction aids in identification of <i>Escherichia coli</i> , <i>Proteus vulgaris</i> ; aids in identification of anaerobes
ONPG	β -Galactosidase	Ester linkage of ortho-nitrophenyl moieties to various carbohydrates; hydrolysis results in release of yellow ortho-nitrophenol	Determines lactose fermentation (yellow color) in slow lactose fermenters; differentiates <i>Neisseria lactamica</i> from pathogenic <i>Neisseria</i> spp.
Oxidase	Cytochrome-c oxidase	Blue compound is produced when tetramethyl- <i>p</i> -phenylenediamine reacts with cytochrome c	Differentiation of nonfermenters; aids in identification of <i>Neisseria</i> , <i>Aeromonas</i> , <i>Vibrio</i> , and <i>Campylobacter</i> spp.
Catalase	Catalase	Breakdown of hydrogen peroxide into oxygen and water, resulting in rapid production of bubbles	Differentiation of staphylococci from streptococci and of <i>Listeria</i> spp. from streptococci
Bile solubility		Autocatalyzes colony in the presence of the surfactant sodium deoxycholate (bile salt)	Presumptive identification of <i>Streptococcus pneumoniae</i> in sputum, blood, and CSF cultures
PYR	PYR	Hydrolysis of amide substrate with formation of free β -naphthylamide, which combines with DMAC to form a bright red color	Identification of group A streptococci; differentiates <i>Enterococcus</i> from group D streptococci
Rapid urease	Urease	Rapid hydrolysis of urea by urease releases ammonia; alkalinity causes phenol red indicator to change from yellow to red	Screening test for <i>Cryptococcus</i> , <i>Proteus</i> , and <i>Klebsiella</i> spp. and <i>Yersinia enterocolitica</i>
Rapid hippurate hydrolysis	Hippuricase	Enzymatic hydrolysis of hippurate visualized by addition of ninhydrin (triketohydrindene)	Positive reaction aids in identification of <i>Streptococcus agalactiae</i> , <i>Campylobacter jejuni</i> , and <i>Listeria</i> spp.
MUG	β -Glucuronidase	Hydrolysis of substrate to fluorescent compound MUG, which fluoresces blue under long-wave UV light	Presumptive identification of <i>E. coli</i> and <i>Streptococcus anginosus</i> group; enterohemorrhagic <i>E. coli</i> is negative
LAP	LAP	LAP hydrolyzes substrate to leucine and α -naphthylamine, which reacts with DMAC to form a red color	Presumptive identification of catalase-negative, gram-positive cocci

CSF, Cerebrospinal fluid; DMAC, *p*-dimethylaminocinnamaldehyde; LAP, leucine aminopeptidase; MUG, 4-methylumbelliferyl- β -D-glucuronide; ONPG, ortho-nitrophenyl- β -D-galactopyranoside; PYR, pyrrolidonyl aminopeptidase; UV, ultraviolet.

Table 9.4 Commercially available manual and automated systems for microbial identification

Name	Manufacturer	Principle	Organisms identified
Manual			
API	bioMérieux ^a	Carbohydrate utilization/ chromogenic substrate	Enterobacterales, other GN bacilli, <i>Staphylococcus</i> spp., <i>Streptococcus</i> spp., <i>Enterococcus</i> spp., <i>Neisseria</i> spp., GP bacilli, yeasts, anaerobes
Crystal E/NF	BD Diagnostic Systems ^b	Carbohydrate utilization/ chromogenic substrate	Enterobacterales, other GN bacilli, <i>Neisseria</i> spp., <i>Haemophilus</i> spp., GP cocci, GP bacilli
MicroLog	Biolog ^c	Carbohydrate utilization	GP organisms, GN organisms, yeasts, anaerobes
Gonocheck	TCS Bioscience ^d	Chromogenic substrate	<i>Neisseria/Moraxella</i> spp.
Micro-ID (RapID)	Thermo Fisher Scientific ^e	Carbohydrate utilization/ chromogenic substrate	Enterobacterales, other GN bacilli, <i>Neisseria</i> spp., <i>Haemophilus</i> spp., streptococci, enterococci, yeasts, anaerobes, UTI, GP bacilli
MicroScan (TouchScan)	Beckman Coulter ^f	Carbohydrate utilization/ chromogenic substrate	GP organisms, GN organisms, UTI, <i>Haemophilus</i> spp., <i>Neisseria</i> spp.
Oxi-Ferm II	BD Diagnostic Systems	Carbohydrate utilization	Nonfermenter GN bacteria
Automated			
BD Phoenix	BD Diagnostic Systems	Carbohydrate utilization/ chromogenic substrate/fluorogenic substrate	Enterobacterales, other GN bacilli, GP
MicroScan (Autoscan, WalkAway)	Beckman Coulter	Carbohydrate utilization/ chromogenic substrate/fluorogenic substrate	Enterobacterales, other GN bacilli, <i>Neisseria</i> spp., <i>Haemophilus</i> spp., streptococci, enterococci, staphylococci, GP bacilli, yeasts, anaerobes
OmniLog	Biolog	Carbohydrate utilization/ tetrazolium violet reduction	GN organisms, GP organisms, anaerobes
Sensititre (TREK)	ThermoFisher Scientific	Carbohydrate utilization/ chromogenic substrate	GN organisms, GP organisms, anaerobes
Sherlock Microbial Identification System	MIDI ^g	Fatty acid analysis of microbial cells	GN organisms, GP organisms, <i>Mycobacterium</i> spp., yeasts
Vitek 2	bioMérieux	Carbohydrate utilization/ chromogenic substrate	Enterobacterales, other GN bacilli, <i>Neisseria</i> spp., <i>Haemophilus</i> spp., streptococci, enterococci, staphylococci, GP bacilli, yeasts, anaerobes
^a Hazelwood, MO. ^b Sparks, MD. ^c Hayward, CA. ^d Buckingham, United Kingdom. ^e Waltham, MA. ^f Brea, CA. ^g Newark, DE. API, Analytical Profile Index; GN, gram-negative; GP, gram-positive; MIDI, Microbial Identification System; UTI, urinary tract infection.			



Fig. 9.11 RapID cartridge containing various substrates. (Courtesy Thermo Fisher Scientific, Waltham, MA.)

Automated identification systems

Several automated identification systems are currently available; most of these systems use turbidity, colorimetry, or fluorescence technology. These systems have a variety of capacities depending on laboratory workflow. Fully automated systems incubate and read the reactions, and computer software interprets the results and provides the identification. An advantage of automated systems includes an interface to laboratory information systems, leading to decreased turn-around times for reporting results. Other advantages include statistical prediction of correct identification, increased data

acquisition and epidemiologic analysis, and automated standardization of identification profiles that can reduce analytical errors. If these systems are used in conjunction with automated susceptibility testing, these data may be linked to the pharmacy for patient management applications. A key application of automated systems is the direct testing of positive blood cultures. Theoretically, early reporting of results can shorten the length of hospital stay, augment therapeutic management of appropriate antimicrobial agents, and thus decrease hospital costs.

Whether using manual multitest (as discussed previously) or automated systems, laboratories use an incubated “purity” plate of the inoculum to accompany the identification procedure. Because many specimens are polymicrobial, a purity plate ensures that the inoculum was pure and was not mixed with another microorganism that would produce erroneous results. With the threat of biological terrorism, automated systems address the rapid, accurate identification of biothreat level A organisms. Many of these organisms, such as *Bacillus anthracis*, *Francisella tularensis*, and *Yersinia pestis*, are rarely seen in clinical microbiology laboratories. Manufacturers of identification systems have responded by including updated data on biothreat level A organisms in their identification databases. However, the identification of some rarely isolated pathogenic bacteria can be inaccurate.

MicroScan systems

The MicroScan System (Beckman Coulter, Brea, CA) consists of plastic standard-size, 96-well microtiter trays in which 32 reagent substrates are included for the identification of bacteria and yeasts. Two systems are available: autoSCAN and WalkAway. Some trays, called *combo trays*, include broth microdilutions of various antimicrobial agents for performing susceptibility tests along with biochemical tests for identification. Some panels are designed for overnight incubation, while the rapid panels provide preliminary results in as soon as 2 hours and final results in 18 hours. MicroScan panels contain dehydrated substrates allowing for room temperature storage and a long shelf life. The wells are inoculated with a heavy suspension of the organism to be tested and incubated at 35° C. Conventional panels are incubated for a minimum of 16 hours to about 20 hours. The autoSCAN system reads standard panels in an automated tray reader that detects bacterial growth or color changes by differences in light transmission. As with other automated systems, identification of the organism is accomplished digitally by collating the readings and matching them to the system’s software database for final identification. The MicroScan WalkAway is fully automated with capabilities to incubate multiple panels (40 or 96 depending on the system), add reagents automatically to conventional panels when required, read and interpret panel results, and print results, all without operator intervention (Fig. 9.12).

In addition to the conventional MicroScan panels, rapid fluorescence panels are available for use in the WalkAway instrument. The rapid panels use fluorescent-labeled compounds to test for preformed enzymes; these panels require only about 2.5 hours for bacterial identification, and the antimicrobial susceptibility is determined in about 7 hours. Fluorometric reactions detect changes in pH because of carbohydrate fermentation. The resultant acid production causes a decrease in pH and a decrease in fluorescence.



Fig. 9.12 WalkAway 96 plus System featuring the test platform and automated data management system. (Courtesy Siemens Healthcare Diagnostics, Tarrytown, NY.)

Studies have indicated that directly inoculating positive blood cultures into MicroScan Combo plates using the WalkAway 96 instrument can yield rapid and accurate results for bacterial identification and antimicrobial susceptibility testing. A study of 1070 positive blood cultures reported that 96.5% of isolates (1033/1070) were identified correctly by the direct method. The antimicrobial susceptibility testing revealed an overall categorical agreement of 92.86% between the direct method and conventional testing.

TREK diagnostic system

The TREK Diagnostic System (Thermo Fisher Scientific) offers two automated systems: the Sensititre Autoreader and the fully automated Sensititre ARIS2X identification system. The Sensititre system uses fluorescence technology to detect bacterial growth and enzyme activity. The system comprises 32 biochemical tests, including selected classic biochemical media reformulated to yield a fluorescent signal. The biochemical test medium, along with an appropriate fluorescent indicator, is dried into the individual wells of the Sensititre plate. Each biochemical reaction is repeated three times in the 96-well plates; each plate is designed to test three separate organisms. All tests are read on the Sensititre Autoreader for the presence or absence of fluorescence. Results are available as soon as 5 hours, although incubation can be extended to overnight if needed. The results are transmitted to a computer for analysis and identification. Plates for antimicrobial susceptibility testing only are also available.

Vitek 2 system

The Vitek 2 system (bioMérieux Inc.) was first introduced in the 1980s. A suspension of the organism is prepared in saline and incorporated into a card. The card is then placed in the reader-incubator module of the instrument, where it is optically scanned and read periodically. The computer software collates the readings and matches them to the database for

final identification. The reader/incubator can accommodate 15, 30, 60, or 120 cards depending on the system. Numerous cards are available for gram-positive cocci, coryneforms, fermentative gram-negative bacilli, nonfermentative gram-negative bacilli, *Haemophilus*, *Neisseria*, and yeasts.

Compared with 16S ribosomal RNA (rRNA) sequencing, the Vitek 2 identified correctly 43 of 50 (86%) anaerobic bacteria to the species level and 47 (94%) to the genus level. In a larger study of 1020 bacterial isolates and 5 fungi, 92.59% (949/1025) isolate identifications matched by the Vitek 2 and the Vitek MALDI-TOF mass spectrometry. Discordant results were identified by 16S rRNA sequencing. Authors reported no errors at the genus level for MALDI-TOF mass spectrometry, whereas the Vitek 2 made 6 (0.58%) errors. Bacteria in the study included typical clinical isolates such as *Pseudomonas* spp., *Acinetobacter* spp., Enterobacterales, and some slow-growing bacteria (*Eikenella corrodens*, *Listeria monocytogenes*, *Haemophilus influenzae*, and others). Another study of over 4000 microorganisms that included gram-positive bacteria, gram-negative bacteria, and yeast found an overall excellent identification agreement between MALDI-TOF mass spectrometry and the Vitek 2.

BD phoenix automated microbiology system

The BD Phoenix Automated Microbiology System (BD Diagnostic Systems) was released in the United States in 2004. Once the 136-well combination panels are inoculated, it is a totally hands-off system that can hold 100 panels: 99 samples and 1 control. Panels are read every 20 minutes for up to 16 hours. Results are generally available in 2 to 12 hours for bacteria and 4 to 15 hours for yeasts. Various colorimetric and fluorometric indicators are used for identification, and a colorimetric redox indicator is used for antimicrobial susceptibility testing. In a study in which 618 isolates were identified by the Phoenix system and MALDI-TOF mass spectrometry, the concordance (agreement) rate was 95.9% at the genus level for nonfermentative gram-negative bacilli and 81.6% at the species level. The concordance rate for gram-positive bacteria was 100% at the genus level and 86.6% at the species level.

Biolog OmniLog ID system

The fully automated Biolog OmniLog ID System is based on the organism's ability to utilize 71 carbon sources and sensitivity to 27 chemical substances. One 96-well plate is used for each organism. The reduction of tetrazolium violet is used as the indicator system. The Biolog database is one of the largest, with more than 2900 species or taxa. Panels are available for aerobic and anaerobic gram-positive and gram-negative bacteria and yeasts. The Gen III OmniLog ID System is a semiautomated system. The Biolog system is not approved for human in vitro testing but is widely used in agricultural and veterinary microbiology laboratories.

Evaluation of identification systems

Most rapid and automated procedures are designed to provide results with greater speed and precision than traditional methods. Many multitest and automated systems are highly accurate and can provide results in 2 to 4 hours, particularly with the Enterobacterales. These systems typically display

lower accuracy with coagulase-negative staphylococci; non-fermentative gram-negative bacilli; and slower-growing, more fastidious bacteria, such as anaerobes.

Whether greater efficiency and productivity are achieved with multitest systems depends largely on the laboratory and the institution or institutions that it supports. Manual and automated systems should be evaluated onsite before changing or augmenting current protocols. The best studies are prospective, side-by-side comparisons of the current in-house or reference procedure with the new system for accuracy, cost-effectiveness, and effect on workflow. Most microbiologists consider 16S rRNA sequencing as the reference method in comparison studies. Automated systems often provide decreased sensitivity and specificity for the identification of biochemically inert bacteria and some fastidious organisms compared with other bacteria. Therefore supplemental and differential media and conventional biochemicals still must be available to support the identification of these organisms. Whether it is a multitest manual method or an automated system, regular updating of the database is critical for accurate identification and correct names of the microorganisms.

POINTS TO REMEMBER

- Phenotyping, serotyping, and genotyping all have important uses in the identification of bacteria.
- Molecular biology assays, such as nucleic acid amplification tests and MALDI-TOF mass spectrometry, provide accurate identification more rapidly than conventional biochemical testing.
- Bacteria can utilize carbohydrates oxidatively, fermentatively, or not at all.
- TSI agar and KIA are useful in determining the ability of bacteria to utilize certain carbohydrates and to produce H_2S .
- The MR and VP tests are used to determine the end products of glucose fermentation.
- Decarboxylase, dihydrolases, and deaminases are enzymes used by bacteria to metabolize amino acids.
- Numerous tests, such as citrate, DNase, indole, nitrate reduction, oxidase, and urease, are important in the identification of gram-negative bacteria.
- Manual multitest systems have improved the identification of bacteria by simplifying inoculation of many different biochemical tests and producing numeric codes that can be compared with numbers in a database.
- Rapid tests often use chromogenic or fluorogenic substrates to assay for preformed bacterial enzymes.
- Automated microbial identification systems offer accurate, rapid identifications with less hands-on time by laboratory scientists.

LEARNING ASSESSMENT QUESTIONS

1. Which of the following tests detects the production of mixed acids from glucose because of subsequent metabolism of pyruvate?
 - a. Methyl red test
 - b. Voges-Proskauer test
 - c. Citrate test
 - d. Indole test

2. The metabolism of glucose to pyruvate by members of the order Enterobacterales is via the Embden-Meyerhof-Parnas pathway. The subsequent metabolism of pyruvate shows this reaction: glucose → pyruvate → acetylmethylcarbinol (acetoin) → 2,3-butanediol. This reaction is the basis for the:
 - a. Oxidase reaction
 - b. Methyl Red test
 - c. Indole test
 - d. Voges-Proskauer test
3. A gram-negative bacillus that produces an acid slant and acid butt on triple sugar iron agar can ferment which of the following carbohydrates?
 - a. Glucose only
 - b. Glucose and lactose or sucrose or both
 - c. Lactose only
 - d. Lactose and sucrose, but not glucose
4. Why is an oil overlay used when testing carbohydrate utilization?
 - a. Minimizes the risk of airborne contamination
 - b. Provides a nutrient source
 - c. Creates an anaerobic environment
 - d. Enhances activity of the pH indicator
5. Tryptophan broth is inoculated and incubated 24 hours. After incubation, Kovac's reagent is added. A red color develops at the surface of the broth. Which product of metabolism was formed?
 - a. Mixed acids
 - b. H₂S
 - c. Phenylpyruvate
 - d. Indole
6. In the citrate utilization test, a positive result is determined by:
 - a. Increase in pH from peptone metabolism
 - b. Increase in pH from urease activity
 - c. Decrease in pH from citrate utilization
 - d. Decrease in pH from nitrate reduction
7. Lysine deaminase:
 - a. Cleaves a carboxy group from lysine
 - b. Cleaves an amino group from lysine
 - c. Adds an amino group to lysine
 - d. Adds a carboxy group to lysine
8. While performing quality assurance on a new lot of triple sugar iron slants, the control organisms gave the following results: *E. coli*, yellow/yellow with gas, *Salmonella* Typhimurium, red/yellow with a black precipitate in the butt. Which of the following statements is true?
 - a. The yellow slant indicates glucose fermentation only.
 - b. The yellow butt of *E. coli* and *Salmonella* indicates galactose fermentation.
 - c. The black precipitate indicates citrate utilization.
 - d. The red slant of *Salmonella* indicates nonlactose fermentation and nonsucrose fermentation.
9. A bacterium is isolated that produces clear colonies on MacConkey agar after 18 hours of incubation. Laboratory testing reveals a positive ONPG, a positive methyl red, and a negative Voges-Proskauer. What do these results indicate about the bacterium's ability to utilize carbohydrates?
 - a. Sucrose fermentation is positive
 - b. Can oxidize lactose but cannot ferment it
 - c. Delayed lactose fermentation
 - d. Uses glucose anaerobically
10. Indole-positive bacteria:
 - a. Produce tryptophanase
 - b. Deaminate lysine
 - c. Decarboxylate tryptophane
 - d. Decarboxylate ornithine
11. How do bacteria that are able to ferment lactose rapidly differ from bacteria that are delayed lactose fermenters?
12. After 24-hour incubation of a nitrate broth with visible growth, *N,N*-dimethyl- α -naphthylamine and sulfanilic acid are added. No color change is noted. What are two possible explanations concerning the reduction of nitrate? What should you do next to determine which explanation is correct?

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Immunodiagnosis of infectious diseases

Donald C. Lehman

CHAPTER OUTLINE

Antibodies in serologic testing, 201

- Antigens, 201
- Acute and convalescent antibody titers, 201
- Monoclonal antibodies, 203
- Antibody specificity and cross-reactivity, 204
- False-negative and false-positive serologic results, 204
- Population studies, 205
- Immune status testing, 205
- Congenital infections, 205

Antigen detection, 205

Principles of immunologic assays, 206

- Precipitation assays, 206
- Agglutination Assays, 206
- Neutralization assays, 210
- Labeled immunoassays, 210

Use of serologic testing in specific diseases, 219

- Serologic testing for syphilis, 219
- Serologic testing for streptococcal infections, 220
- Serologic diagnosis of viral diseases, 220
- Serologic diagnosis of fungal infections, 221

Direct antigen detection assays, 222

- Streptococcal pharyngitis, 222
- Bacterial meningitis, 223
- Giardiasis and cryptosporidiosis, 223
- Infections in immunocompromised patients, 224

Bibliography, 226

OBJECTIVES

After reading and studying this chapter, you should be able to:

1. Define *antigen*.
2. Compare the structure and function of immunoglobulin G and immunoglobulin M.
3. Discuss the importance of acute and convalescent antibody titers.
4. Interpret significant antibody titer results.
5. Compare polyclonal and monoclonal antibodies.
6. Explain the causes of false-negative and false-positive immunologic test results.
7. Justify screening women who are considering pregnancy for rubella antigens.
8. Rationalize the absorption of biotin from clinical specimens before performing a biotin/streptavidin immunoassay.
9. Justify using an enzyme immunoassay instead of an indirect fluorescent assay to detect antibodies
10. Recognize the significance of serologic tests in the following situations:
 - Population studies
 - Immune status testing
 - Congenital infections
 - Infections beyond the newborn period
11. Describe the principles and applications of each of the following immunologic methods:
 - Precipitation assays
 - Agglutination assays
 - Neutralization assays
 - Immunofluorescent assays
 - Enzyme immunoassays
 - Complement fixation (CF)
 - Western blot
 - Dot blot
12. Compare direct agglutination, passive agglutination, and reverse passive agglutination.
13. Describe the clinical applications of antibody detection for the following diseases:
 - Syphilis
 - *Streptococcus pyogenes* infection
 - Infectious mononucleosis
 - Viral hepatitis
 - Human immunodeficiency virus (HIV) infection
 - Fungal infections

14. Describe the current clinical applications of direct antigen detection methods in each of the following infectious diseases:

- *Streptococcus pyogenes* infection
- Respiratory tract viral infections
- Bacterial meningitis
- Giardiasis and cryptosporidiosis
- HIV infection
- Infections in immunocompromised patients

KEY TERMS

Acute phase	Hybridoma
Anamnestic immune response	Immune complex
Antibody titer	Immunodiffusion
Antigens	Immunogen
Avidity	Indirect agglutination
Biotin-streptavidin systems	Indirect fluorescent antibody (IFA)
Chemiluminescent immunoassay (CLIA)	Indirect sandwich immunoassay
Coagglutination	Monoclonal antibody
Competitive immunoassays	Polyclonal antibody
Complement fixation (CF)	Postzone
Conjugate	Precipitation
Convalescent phase	Prozone
Cytopathic effect (CPE)	Radial immunodiffusion
Direct fluorescent antibody (DFA)	Radioimmunoassay (RIA)
Direct sandwich immunoassay	Reagin antibodies
Double immunodiffusion	Reverse passive agglutination
Epitopes	Rheumatoid factor (RF)
False-negative	Seroconversion
False-positive	Serology
Fluorochrome	Specificity
Heterophile antibodies	Zeta potential
	Zone of equivalence

Case in point

A previously healthy, 33-year-old male patient presents to his physician with an unintended 10-lb weight loss over a 3-month period, intermittent diarrhea for several months, and a fever of unknown cause. A complete blood count revealed a slightly elevated white blood cell (WBC) count. The WBC differential was 72% neutrophils, 15% monocytes, 10% lymphocytes, and 3% eosinophils. Based on these findings and the clinical presentation, the physician ordered a blood test for antibodies directed against human immunodeficiency virus (HIV). The result of the screening test was reactive.

Issues to consider

After reading the patient's case history, consider:

- The importance of the patient's case history and laboratory results
- The significance of the blood test for HIV
- What additional test should be performed next
- The role of cultures and direct antigen detection in the diagnosis of infectious diseases

The phenomenon of bacterial agglutination in human sera was discovered in 1896 and was quickly recognized as a powerful tool by bacteriologists. Not only could sera be used to identify and differentiate bacteria (serotyping), but also the sera from infected patients could be tested for the ability to agglutinate a known microorganism. The reaction of patients' sera with bacteria was indicative of prior exposure to the organism and of the level of immune response and protection against the infectious agent. The proteins in human sera causing bacterial agglutination, called *antibodies*, form because of infection by microorganisms.

Serology (literally "the study of serum") is the study of antibodies and their reaction with **antigens**, molecules that bind specifically to antibodies or T-cell receptors, in the diagnosis of infectious diseases. These tests originally referred to the use of serum or plasma, but now several body specimens including cerebrospinal fluid (CSF) and urine are used. Fluorescent immunoassays can also be performed on tissue. Immunologic assays based on the principles of antibody-antigen reactions are also used to detect and quantify analytes, such as serum proteins, hormones, and drugs, in clinical chemistry and forensic science. Immunoassays are used in several point-of-care tests and home test kits.

Perhaps the greatest advantage of serologic testing is that it is an excellent means for detecting infectious agents that are either difficult or impossible to culture. Certain infectious agents, such as viruses or some sexually transmitted bacteria, are difficult to culture, usually because of either their specific growth requirements or previous antimicrobial therapy that decreases the number of viable organisms. Many fungi and mycobacteria also take an extremely long time to grow. In addition, many organisms are not grown because of significant risk to laboratory scientists. Serologic tests can be used not only under these conditions but also for prognostic information and in the monitoring of therapy, such as with chronic hepatitis.

As powerful as immunologic tests are, using serologic procedures in diagnosis has disadvantages. The most significant problem is that to measure the host immune response to an organism, 10 to 14 days must pass after the onset of an infection. In some cases (e.g., HIV, hepatitis B virus [HBV], and hepatitis C virus [HCV]), weeks to months must pass before antibody levels are detectable. Immunocompromised patients can have an antibody response that is either diminished or nonexistent, impairing the ability to use any serologic procedure. Also, the antibody being detected may have been produced against another organism, and a **false-positive** test is observed.

The discovery that immune hemolysis could be mediated by anti-erythrocyte antibodies in the presence of complement and could be titrated added a new approach to the diagnosis of infectious diseases. The serum of patients could be examined for the presence of antibodies that do not agglutinate or precipitate their respective antigens, but activate, or fix, complement. This serologic method, known as **complement fixation** (CF), is a very sensitive method for detecting antibodies to their corresponding antigens.

The first practical laboratory tests for detecting newly discovered infectious agents are still generally based on serology. The types of tests available continue to increase, allowing the diagnosis of many new infectious diseases. For example, acquired immunodeficiency syndrome (AIDS), viral hepatitis,

and Lyme disease are most frequently diagnosed in the laboratory with serologic tests. These three diseases all are caused by infectious agents that are difficult to cultivate in laboratories. Although currently available serologic tests for these and other agents are not perfect, improvements have resulted from advances in preparation of antibodies and antigens of high purity. A wide variety of high-quality commercial kits are available. Immunologic tests also are used to detect antigens that are detectable earlier than antibodies; however, they are not available for as many organisms as the classic antibody detection tests. This chapter discusses the aspects of using immunologic assays to diagnose infectious disease by detecting either antibodies or antigens in patient samples.

Antibodies in serologic testing

Antigens

Antigens are relatively high-molecular-weight substances, usually proteins or polysaccharides (less commonly, lipids or nucleic acids either alone or complexed to proteins or polysaccharides), that combine specifically with antibody molecules. Antigens that are recognized as foreign substances by a host are said to be *immunogenic*, capable of eliciting an immune response. Often, the terms *antigen* and **immunogen** are used interchangeably; the former term refers to the antibody-binding properties of the molecule, whereas the latter refers to the antibody-eliciting property of the molecule.

The typical immune response to a large complex antigen, such as a bacterium or a virus, results in the stimulation of many different lymphocytes with differing **specificity** to the available antigenic determinants (**epitopes**). Chapter 2 provides an explanation of antibody formation. Most natural antigens are extremely large. Therefore the response generated to these molecules is polyclonal, resulting in a mixture of antibodies recognizing different epitopes with different binding affinities. A **polyclonal antibody** is composed of a mixture of antibody molecules derived from multiple cells against multiple epitopes found on an antigen or directed against different antigens altogether. For instance, when an animal or person is exposed to any bacterium, different antibody molecules are made to each of the numerous epitopes found on different antigens on the bacterium.

Microorganisms contain a wide array of molecules capable of eliciting an immune response in the host. Immunogenic substances may be structural or nonstructural components of the microorganism. For example, many pathogenic bacteria have polysaccharide capsules that coat the organism. Because these capsules are located on the cell surface, they are often the first antigenic determinants recognized by the host. Additional structural antigenic components are the bacterial cell wall, membrane proteins, and pili proteins. Nonstructural antigens include bacterial enzymes and toxins that may be found within the cell or are released into host tissues. Because many bacterial components are “hidden” within the cell, antibody responses to some of them may not develop until the cell has been degraded by the host’s innate immunity (e.g., phagocytic cells, complement). A single infecting bacterial species typically stimulates the production of numerous antibodies with different specificities.

Acute and convalescent antibody titers

In serologic assays, a patient’s serum is tested for antibodies reactive to specific antigens taken from an infectious agent. If the antibodies are present, this indicates an infection at some point in the patient’s life. It is possible to semi-quantitate the amount of antibody by making serial twofold (doubling) dilutions of the serum, producing progressively lower concentrations of antibody (e.g., 1:2, 1:4). A standard amount of antigen from an infectious agent is mixed with each antibody dilution, and the antibody-antigen reactions are detected in some manner. A positive result is reported as an **antibody titer**, which is the reciprocal of the highest dilution of serum showing reactivity. However, this type of serial dilution method is not very precise, and the difference of only one tube dilution is not considered significant in determining the presence of a recent infection. Antibody tests for a wide variety of infectious agents are commercially available (Table 10.1). Antigen-antibody (**immune complex**) detection methods, along with specific diagnostic applications, are discussed later in this chapter.

An individual’s first exposure to an infectious agent primarily results in the production of immunoglobulin M (IgM). Subsequent exposure to the same antigen elicits a secondary

Table 10.1 Examples of commercially available serologic tests for diagnosis of infectious disease

Type of infection	Serologic test methods available
Bacterial	
<i>Bordetella pertussis</i>	EIA
<i>Borrelia burgdorferi</i>	EIA, IFA, WB
<i>Helicobacter pylori</i>	EIA
<i>Legionella pneumophila</i>	EIA, ICT, IFA
<i>Mycoplasma pneumoniae</i>	EIA, ICT, IFA
<i>Streptococcus pyogenes</i>	NT, QN
<i>Treponema pallidum</i>	IFA, EIA, flocculation
Fungal	
<i>Histoplasma capsulatum</i>	CF, ID, LA
Parasitic	
<i>Toxoplasma gondii</i>	EIA, IFA, LA
Viral	
Cytomegalovirus	EIA, IFA
Epstein-Barr virus	CIA, EIA, ICT, IFA, LA, WB
Hepatitis viruses A, B, and C	CIA, EIA
Herpes simplex virus	EIA, ICT, IFA, WB
HIV types 1 and 2	CIA, EIA, ICT, IFA, WB
Influenza viruses A and B	CF, EIA, HAI
RSV	CF, EIA, IFA
Rubella virus	EIA, HAI, LA, WB

CF, Complement fixation; CIA, chemiluminescence; EIA, enzyme immunoassay; HA, hemagglutination; HAI, hemagglutination inhibition; HIV, human immunodeficiency virus; ICT, immunochromatographic test; ID, immunodiffusion; IFA, indirect fluorescent antibody; LA, latex agglutination; NT, neutralization; QN, qualitative nephelometry; RSV, respiratory syncytial virus; WB, Western blot.

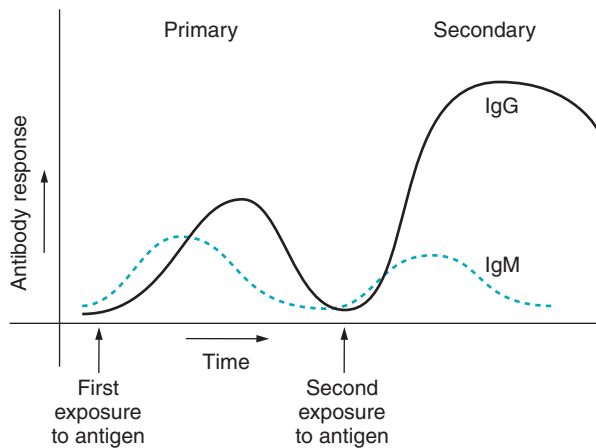


Fig. 10.1 Primary and secondary antibody responses.

or **anamnesic immune response**, which is characterized by a rapid increase in immunoglobulin G (IgG) associated with higher levels, a prolonged elevation, and a more gradual decline (Fig. 10.1). IgM antibody synthesis plays a minor role in a secondary immune response. Serologic tests that are designed to detect IgG and IgM antibodies separately take advantage of the differences in IgM production between a primary and a secondary immune response. A positive test result for IgM antibody is considered suggestive of a primary, current, or very recent infection, whereas the presence of IgG antibody alone suggests a previous infection or exposure. Interpretation of high IgM antibody titers must be made cautiously. In some infectious diseases, high IgM levels can persist beyond the acute state. Alternatively, a negative IgM titer does not exclude an infection because it can sometimes take several weeks after onset of an infection before IgM will be detectable.

Similarly, the presence of significant levels of IgM antibody (with or without IgG) in a newborn suggests an in utero infection. IgM can be synthesized by the fetus and cannot cross the placenta from mother to fetus, whereas the presence of IgG antibody only in the newborn is indicative of passive maternal transfer of IgG across the placenta, not in utero infection.

Several methods are available for removal or inactivation of serum IgG, allowing for preferential detection of IgM. One of the most common methods for physical removal of IgG uses commercially available miniature ion-exchange chromatography columns to trap IgM, while allowing IgG to be washed through with a buffer solution. The IgM antibody is collected by elution from the column with a lower pH buffer. Another method of removing IgG from whole serum uses adsorption to protein A found in the cell wall of staphylococci. Protein A binds most subclasses of human IgG, facilitating their removal. Also, anti-human IgG inactivates or removes (requires precipitation and centrifugation) IgG for IgM-specific assays. This method has the additional advantage of removing **rheumatoid factor** (RF) that would bind to the IgG–anti-IgG complexes. RF is present in cases of rheumatoid arthritis and some other autoimmune disorders.

It is sometimes practice to test a single serum specimen for IgG antibody to attempt to diagnose a recent or past infection. In many cases, the presence of IgG antibody is difficult or impossible to interpret; it may represent a past infection,

either clinically apparent or subclinical (without overt symptoms). A relatively high titer of IgG does not necessarily indicate a recent infection. Antibody levels vary based on host genetics, age, comorbidities, and the immunogenicity of the infectious agent. A high antibody titer simply means that the host is a high responder to that specific antigen and has a history of infection. However, in certain cases, such as infections that are rare (e.g., rabies) or have been present for a relatively long time (chronic) when symptoms first appear (e.g., AIDS), the presence of IgG antibody in a single serum specimen might be diagnostic. IgG and IgM testing should be performed concurrently.

Unless a serologic test is designed to measure IgM-specific and IgG-specific antibody in a single serum specimen, serologic diagnosis of an infectious disease requires measurement of total antibody concentration in both **acute-phase** and **convalescent-phase** serum specimens. Specific IgG antibody is usually not detected in serum collected during the acute phase of the illness (within 1 week of manifestation of symptoms). A significant increase in IgG detected during the convalescent (recovery) phase, usually 2 weeks later, is diagnostic for infection and results from class switching from IgM to IgG.

Because some IgG antibody may already be present in a patient's acute-phase serum specimen, it is generally necessary to quantify the concentration of antibody in both the acute-phase and the convalescent-phase specimens. In acute and convalescent antibody titer testing, sera are generally tested as twofold dilutions in a series of tubes. A fourfold increase (two doubling dilutions) in total antibody titer between acute-phase (acute antibody titer) and convalescent-phase (convalescent antibody titer) sera is considered diagnostic for a current infection. It is important that both sera be tested at the same time because most serologic tests have an inherent variability that can alter the titer by at least twofold. Testing the sera at the same time reduces this variability.

Disadvantages of acute and convalescent serum testing include return visits for the patient to have blood drawn and the time needed to detect an increase in antibody titer. In addition, false negatives are possible if sera are collected too early in the course of the disease. In this case, an acute-phase sample would be negative, and the convalescent-phase sample would be positive. This scenario is referred to as **seroconversion**. Although seroconversion usually occurs within 2 to 3 weeks after onset of illness, it may be delayed in certain patients or types of infection.

Some commercial diagnostic tests are designed without the need to perform serial dilution and establishment of titers. Instead, undiluted serum or a single serum dilution is tested. In these situations, the determination of a single positive result suggests, but does not always prove, current infection. Generally, if serologic diagnosis of current infection on a single serum specimen is to be attempted, an IgM assay as well as an IgG-specific assay should be performed. If both assays are negative, the patient probably has not been infected. If both assay results are positive or just IgM antibody is detected, the patient probably has been infected recently. If just IgG antibody is present, the patient probably has been infected in the past and does not have a current infection. It is very important that laboratory scientists completely understand all of the performance characteristics of a commercial serologic test system.

Monoclonal antibodies

When an animal is exposed to a single antigen, antibodies to different epitopes on the antigen can be produced. These antibodies are called *polyclonal antibodies* because each antibody was derived from a different clone of plasma cells. In serologic testing, antibodies can be used to detect antigens in patient samples. The problems with polyclonal antibodies are low specificity because the antibody solution contains multiple antibodies with different specificities, which can result in a high level of cross-reactivity.

The problems with a polyclonal antibody can be avoided with the use of a **monoclonal antibody**. This antibody is derived initially from one cell that has been exposed to one epitope. This cell divides, producing a clone of identical cells, and then produces an antibody specific to this one epitope. Monoclonal antibodies are rarely naturally occurring and are usually associated with some type of abnormal immune disease process. The production of monoclonal antibodies in the laboratory has become commonplace. It is used extensively in serologic testing in which

the identification or quantification of an antigen is the primary goal. These antibodies have strong binding kinetics to specific epitopes on antigens (**avidity**) and can discriminate among closely related antigenic determinants (specificity). However, because monoclonal antibodies are very specific, they have low sensitivity. If a bacterial population contains an altered or mutated form of an antigen, it may not react to the monoclonal antibody, resulting in a **false-negative** test result.

Monoclonal antibodies are purified from a single clone of cells. In 1975, George Köhler developed the technique for producing monoclonal antibodies in the laboratory. In this method, a scientist injects a crude antigen mixture into a mouse or other animal and then selects a clone of plasma cells that produces a specific antibody against an antigen. This process of producing a monoclonal antibody can take 3 to 6 months. A single antibody-producing lymphocyte is fused to a myeloma cell to form a hybrid. This hybrid proliferates to form a cell line called a **hybridoma** (Fig. 10.2). Monoclonal antibodies have also been used to treat infectious diseases such as Ebola and coronavirus disease 2019.

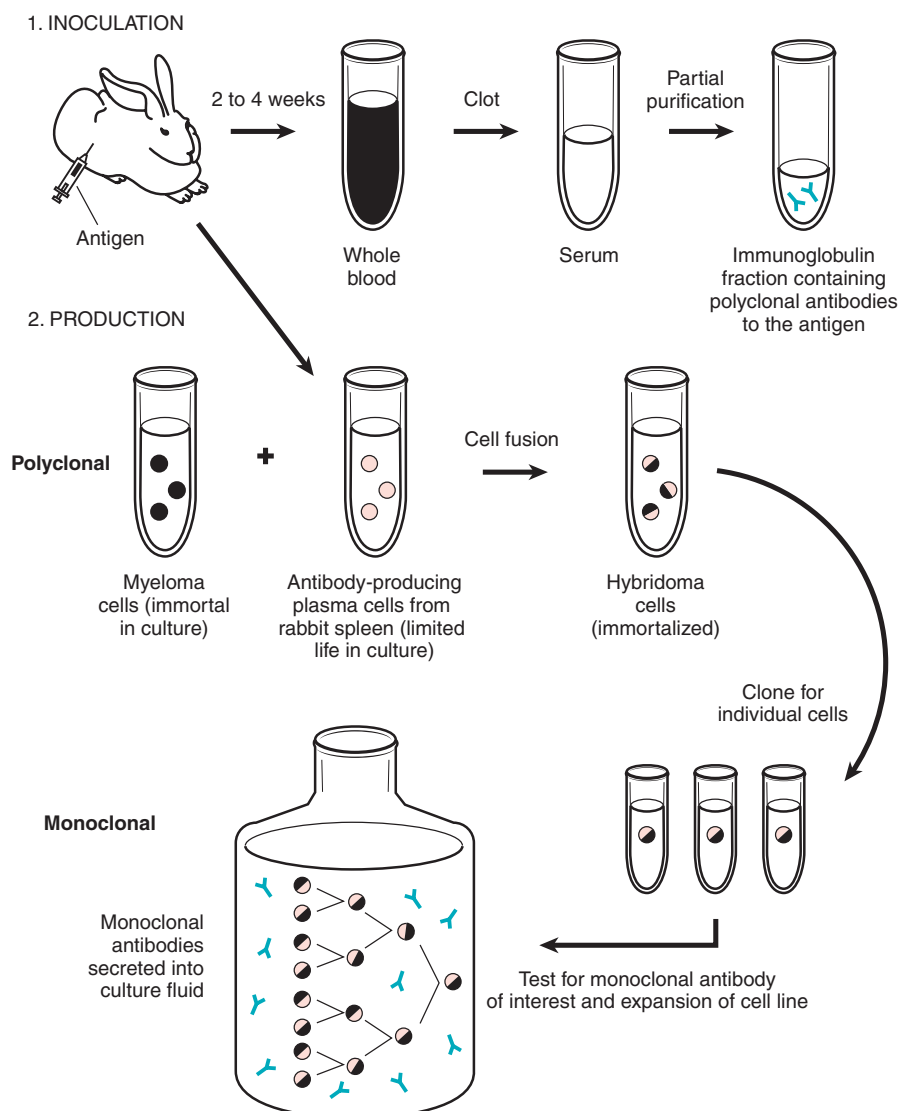


Fig. 10.2 Preparation of polyclonal and monoclonal antibodies.

Antibody specificity and cross-reactivity

Most antigen-antibody reactions show high specificity; that is, the antigen-binding sites on the antibody molecule react with specific epitopes and not with other antigens containing different epitopes. However, some antigens from different microorganisms are similar, and a host may respond by producing antibody, not only to the invading organism but also to antigenically similar organisms. These antibodies are said to *cross-react*, which may lead to misinterpretation of serologic tests. Antibodies produced in response to one molecule that also react against an antigen from an unrelated source are called **heterophile antibodies**. Because of antibody cross-reactivity, it is often best to perform a battery of serologic tests using the organisms known to show cross-reactivity and to do so with paired sera. An example of this type of testing would be a fungal antibody panel consisting of tests for histoplasmosis, blastomycosis, and coccidioidomycosis.

False-negative and false-positive serologic results

An ideal serologic test would have 100% diagnostic accuracy; that is, it should be positive on all specimens from patients with the infection and negative on all specimens from patients without the infection by the specific agent (Fig. 10.3A). In practice, serologic tests, like other laboratory tests, fall short of this ideal. The accuracy of diagnostic tests is measured in terms of *sensitivity*, *specificity*, *positive predictive value*, and *negative predictive value*. See Chapter 5 for a discussion of these terms.

A false-negative serologic test is defined as a negative result for a patient who really is infected. It may occur for many reasons. A patient may be immunocompromised and unable to respond to an immunogenic stimulus. This might be the case in an individual with a congenital or acquired immunodeficiency disease or in a patient receiving either immunosuppressive therapy after organ transplantation or cancer chemotherapy. In addition, neonates may not always respond to an infectious agent because of an immature immune system. For some infections, such as Lyme disease and Legionnaires' disease, antibody titers may not increase until months after acute infection, leading to false-negative serologic test results on serum samples collected too early in the course of the infection. Not all individuals react the same way to immunogenic stimuli because of the inherent genetic differences in the immune system among individuals.

A different type of false-negative result may occur in assays designed specifically to detect IgM antibody. It might be a result of competition for antigen-binding sites between IgG and IgM antibodies in a serum specimen from a patient with high levels of IgG and relatively low levels of IgM (see Fig. 10.3B). IgM antibody tests are sometimes performed on the serum of a newborn to diagnose an in utero infection. A newborn's serum specimen might contain maternal IgG to a specific infectious agent as well as low levels of fetal IgM. The IgG antibody molecules bind to antigen in the assay and block IgM binding, producing a false-negative IgM result. Most assays designed to detect

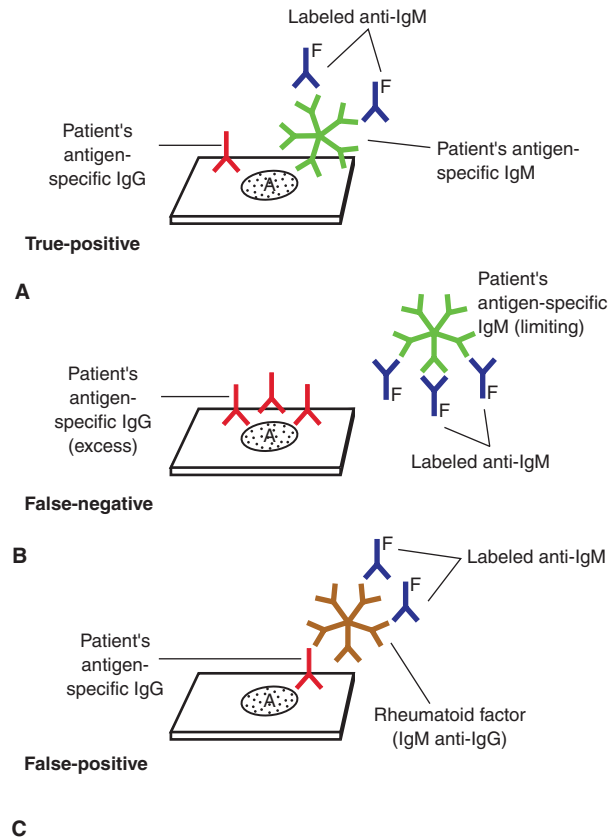


Fig. 10.3 Causes of false-positive and false-negative immunoglobulin M (IgM) assays. **A**, True-positive IgM assay. **B**, False-negative IgM assay. Excess antigen-specific immunoglobulin G (IgG) inhibits antigen-specific IgM from binding. **C**, False-positive IgM assay. Rheumatoid factor (RF) binds to antigen-specific IgG and is detected with labeled anti-IgM antibody. A, Antigen; F, fluochrome or another label.

IgM antibody include some initial procedure to separate IgG physically from IgM or to “capture” IgM in the assay and then remove IgG.

A false-positive serologic test result is a positive result for a patient who is not infected by the specific agent for which the test is designed. It might occur from the production of cross-reacting antibody, as discussed previously, or from reactivation of a latent organism resulting from infection by a different organism. Influenza A virus infection can cause reactivation of latent cytomegalovirus (CMV) with a concomitant increase in CMV antibodies. False-positive IgM antibody assays can also occur. These are caused by the presence of RF activity in the serum. RF is generally an IgM antibody that is produced in some individuals and binds to the Fc region of the individual's own IgG. IgM RF cannot be readily differentiated from organism-specific IgM in some serologic tests. If both organism-specific IgG and RF (but not organism-specific IgM) are present in a patient's serum specimen, the serologic test result may be falsely positive for an organism-specific IgM antibody (see Fig. 10.3C). Finally, individuals receiving intravenous immunoglobulin, a product prepared by pooling large quantities of plasma from multiple volunteer donors, may show a specific antibody to various infectious agents because of passive transfer, not active infection. Laboratory personnel

must be aware of this possibility and any therapy that may be of significance in interpreting serologic test results for a specific patient specimen.

Population studies

Serologic tests for a specific infectious agent or a panel of agents may be performed to determine the percentage of individuals previously exposed or infected with the agents in a geographic area. This information provides epidemiologists and public health officials with information about how widespread an infectious agent is in a given area. Such studies have shown that the fungus *Histoplasma capsulatum*, which causes histoplasmosis, is widely distributed in the Ohio River Valley and that the bacterium *Borrelia burgdorferi*, which causes Lyme disease, is common in the upper Midwest, New England, and Middle Atlantic states. Similarly, serologic studies performed on animals that are reservoirs for human disease may alert public health officials that a disease (e.g., West Nile virus) may be active in the area and may pose a threat to humans.

Immune status testing

In several situations, it may be important to determine whether an individual is immune, through either previous infection or immunization, to a specific infectious disease. For example, women considering pregnancy should be screened for rubella and CMV antibodies; both infections can cause morbidity or mortality to the fetus. It is generally recommended that all women of childbearing age be tested for their rubella immune status; if it is negative, they should be vaccinated before considering pregnancy.

An additional situation in which immune status testing might be considered is organ or bone marrow transplantation. CMV may reside latently in leukocytes of donor tissue and can cause significant disease in a nonimmune recipient. It is recommended that a CMV-negative transplant recipient receive tissue and blood products from a CMV-negative donor.

Congenital infections

Serologic testing is often used to help diagnose congenital infections (acquired in utero) in a newborn; some infectious agents can cross the placenta and cause fetal infections. Such infections might cause only minimal symptoms in the mother during pregnancy and may go undiagnosed at the time. However, if the mother is nonimmune, the infection can be significant to the fetus. The agents most commonly found are the TORCH agents: *Toxoplasma gondii*, which causes toxoplasmosis; other (*Treponema pallidum* subsp. *pallidum*, which causes syphilis; parvovirus B19); rubella virus; CMV; and herpes simplex virus.

Testing the mother's serum for IgG antibody is useless unless she was screened prenatally or early in pregnancy and antibody test results were negative. Because the TORCH agents are common infectious agents, many individuals have IgG antibody remaining from previous infections. Similarly, testing for IgG antibody in the newborn is of no value because maternal IgG crosses the placenta and is present in neonatal serum. Testing for maternal IgM antibody is likewise of little value (unless infection occurred near the time of delivery)

because it may be undetectable 1 or 2 months after infection. Therefore IgM antibody detection on neonatal serum is the method of choice for serologic diagnosis of congenital infection by one of the TORCH agents.

Antigen detection

Antibody detection can require a significant length of time to become positive. Direct antigen-based tests are generally considered to be less sensitive than culture; however, they can yield diagnostic information much sooner, many within 30 minutes. This shorter time is extremely important in treatments that require documentation of the presence of a specific agent.

Numerous infectious agents produce antigens that can be identified from almost any body fluid (serum, urine, sputum, CSF, or exudates) or cellular material. The modern era of direct antigen detection began in the mid-1970s. The capability to produce large quantities of highly specific monoclonal antibodies led to a sharp increase in production of rapid diagnostic assays. Direct microbial antigen detection is the process by which microbial antigens, such as capsular polysaccharide or cell wall components, are identified in specimens obtained from an infected host. These antigens can be recognized by and combine specifically with antibody molecules to form stable immune complexes.

The process of antigen detection involves mixing a clinical specimen with an antibody preparation specific for the antigen of interest. If the microbial antigen is present in the specimen, an antigen-antibody interaction occurs, forming an immune complex. This complex can be detected by many techniques. Some methods take advantage of the fact that antibody molecules are polyvalent—they have two or more antigen-binding sites—and can combine with two or more antigens (Fig. 10.4). Many techniques use indicator molecules such as enzymes and substrate, **fluorochrome** excitation, or chemiluminescence. The tests used to detect these proteins (antigens) are similar to the tests used in the detection of an antibody.

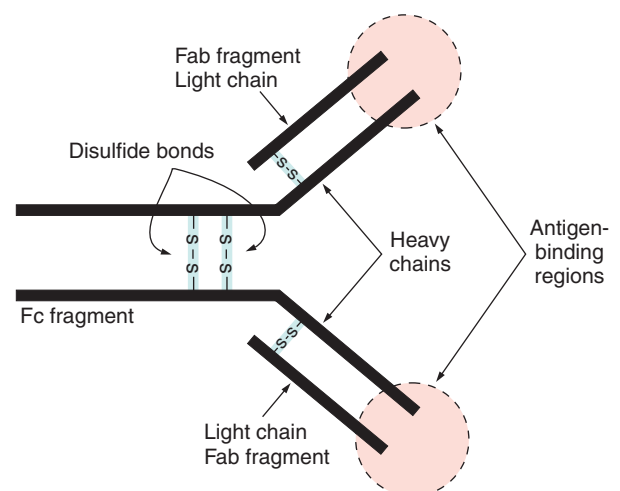


Fig. 10.4 Structure of an antibody monomer.

Principles of immunologic assays

Precipitation assays

Precipitation reactions involve the diffusion of soluble antigen and antibody. At a critical point, when the concentrations are optimal, a visible precipitate forms that is composed of an insoluble complex of antigens and antibodies. Precipitation reactions are most stable when performed in an agarose gel; however, some are conducted in liquid. Precipitation methods with diagnostic significance for infectious diseases are **double immunodiffusion**, **radial immunodiffusion**, and **flocculation** tests.

Double immunodiffusion

Immunodiffusion refers to the movement of antigens and/or antibodies toward one another. In the *double immunodiffusion* or *Ouchterlony gel diffusion* (Fig. 10.5) technique, cylindrical holes or wells are cut out of an agarose gel and spaced appropriately in a small Petri dish. For antibody detection, a known crude or purified antigen extract of a microorganism is placed in one well of the agar plate, and a patient's serum sample is added to a nearby well. The antigen and antibody molecules in solution diffuse out of the wells and through the porous agarose. If antibody specific for the antigen is present, the two components combine at a point of optimal concentration called the **zone of equivalence** and produce a visible precipitin band, or line

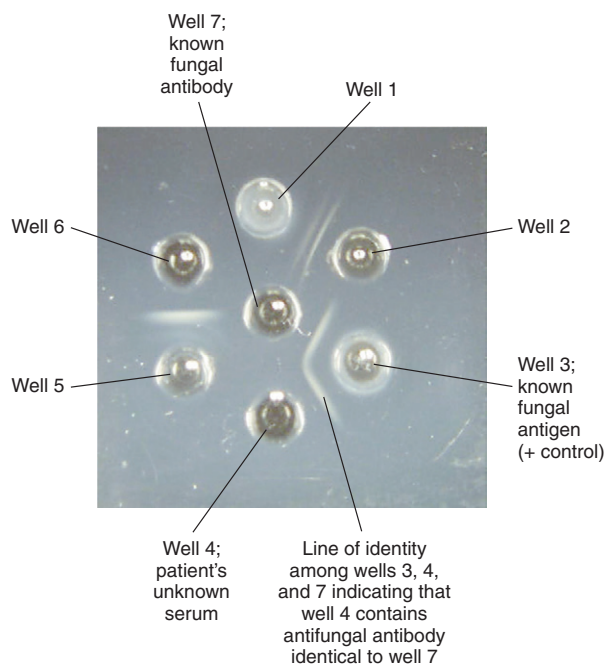


Fig. 10.5 Ouchterlony plate. A serum specimen containing an unknown antibody is placed in one well (e.g., well 4), a known antigen of interest is placed in an adjacent well (well 3—positive control), and a known antiserum to the antigen is placed in another adjacent well (well 7—positive control). The antigen and antibody molecules in the solution diffuse from the wells and through the porous agarose. If the unknown serum contains antibody to the known antigen, a precipitin band forms at a point of optimal concentration of each component. This precipitin band is called a *line of identity*.

of precipitation. Because the test relies on passive diffusion of molecules, diffusion is slow and is not generally amenable to rapid diagnosis. Reactions may take 48 to 72 hours to develop. It is most commonly used to detect fungal exoantigens or serum antibodies. Double immunodiffusion is primarily limited to detecting antibodies to fungal pathogens, including *H. capsulatum*, *Blastomyces dermatitidis*, and *Coccidioides immitis*.

Single radial immunodiffusion

With single radial immunodiffusion, known antibody is evenly distributed in agar placed into a plastic dish. Wells are cut in the agar, and a sample is added to the wells that may contain antigen recognized by the antibody. The antigen passively diffuses through the agar and forms a precipitate ring at the zone of equivalence with the antibody. The diameter of the zone of precipitation is directly proportional to the concentration of the antigen. This technique can be used to quantify antigen concentration, but it is not used much today, having been replaced by quicker methods.

Flocculation

Flocculation tests are a variation of precipitation tests that also have some properties of agglutination assays. In these tests, because of the chemical nature of the antigen (not truly soluble), the antigen-antibody reaction forms a microscopically visible clump or precipitate of fine particles that remain in suspension. The most important applications for flocculation tests in antibody detection relate to syphilis serology. Following infection with *T. pallidum*, the body reacts by developing two classes of antibodies: (1) specific or treponemal antibodies directed against *T. pallidum* antigens and (2) non-specific or nontreponemal antibodies directed against normal host tissue. These nontreponemal antibodies, also referred to as **reagin antibodies**, are detected by flocculation reactions. Syphilis serology is discussed later in this chapter.

Nephelometry

Nephelometry has recently gained popularity as a sensitive method to detect immune complexes formed in liquid. This method is based on the light-scattering ability of insoluble particles. A beam of light, incident light, is passed through a tube containing the mixture. The amount of scattered light is measured at an angle, typically 90 degrees, to the incident light. The scattered light is directly proportional to the concentration of immune complexes. By using standards, it is possible to quantify the immune complexes, referred to as *quantitative nephelometry*. A special instrument, a nephelometer, is required. The method can be applied to measuring an antibody or an antigen and analytes, such as C-reactive protein.

Agglutination assays

Agglutinating antibodies react with antigens on the surface of microscopic particles to form visible clumps. Agglutination tests can be performed in test tubes, but they are generally performed on the surface of glass, plastic, or cardboard slides. Agglutination tests for antibody detection can be classified as either direct (natural carrier particle) or indirect (artificial carrier particle) agglutination. **Indirect agglutination** is also

referred to as *passive agglutination*. **Reverse passive agglutination** uses an antibody attached to a particle to detect an antigen. In contrast with precipitation reactions, in agglutination reactions, either the antigen or the antibody is bound to a particulate carrier (making it insoluble) before forming an immune complex.

Serologic tests based on direct particle agglutination take advantage of antigens that naturally occur on biological cells. Most commonly, these cells are whole bacteria or erythrocytes. In whole bacterial cell agglutination, surface antigens found on the bacterial cell wall or capsule allow cross-linking and macroscopic agglutination of the cells in the presence of a specific antibody. Serotyping enteric pathogens such as *Salmonella* is based on the detection of bacterial surface antigens using commercially prepared sera. Direct particle agglutination of erythrocytes is used in immunohematology to determine an individual's blood type.

Latex agglutination

Various antigens can be passively or chemically coupled to naturally occurring particles like erythrocytes or to synthetic particles such as latex (polystyrene) beads. These beads, usually about 1 μm in diameter, can be a variety of colors and enhance the visibility of agglutination reactions. In this arrangement, the particle can be agglutinated by specific antibody found in a patient's serum specimen. The antigen-coated latex beads form a homogeneous milky suspension, which, when mixed with a specimen containing a specific antibody, results in antigen-antibody binding. However, this primary binding between antigen and antibody molecules does not produce a visible agglutination (clumping) reaction. The antibody must interact with the antigen on two different latex beads (secondary binding) to produce agglutination.

In reverse passive agglutination, each latex bead can contain thousands of monoclonal or polyclonal antibody molecules. When mixed with a specimen that contains antigens specific for the antibody on the latex beads, antigen-antibody binding results. However, antigen molecules are cross-linked by the antibody-coated latex beads only if the antigen molecules contain multiple epitopes, as is common for high-molecular-weight polysaccharides, proteins, or microorganisms. This secondary cross-linking of antigen and antibody molecules results in a macromolecular lattice and produces a visible agglutination reaction (Fig. 10.6).

Latex agglutination (LA) assays are generally conducted on a treated cardboard or glass slide using a liquid specimen and volumes of about 50 μL for both the clinical specimen and the latex bead suspension (Fig. 10.7). The specimen and latex suspension are mixed thoroughly, and the slide is rocked or rotated by hand or with a mechanical device for 2 to 3 minutes before reading with appropriate lighting and the naked eye. The reaction depends on many variables; therefore standardization is imperative. Variables include latex particle size, avidity of the antibody, isotype of the antibody (IgM or IgG), reaction temperature, pH, ionic strength of the solution, and concentration of antigen in the specimen. IgM is a pentamer and is a more effective agglutinin than IgG. Microbial polysaccharide or protein can be detected at levels of 0.1 ng/mL.

The strength and rapidity of the reaction vary depending on the test conditions and, most importantly, on the antigen concentration in the specimen. With a high antigen concentration

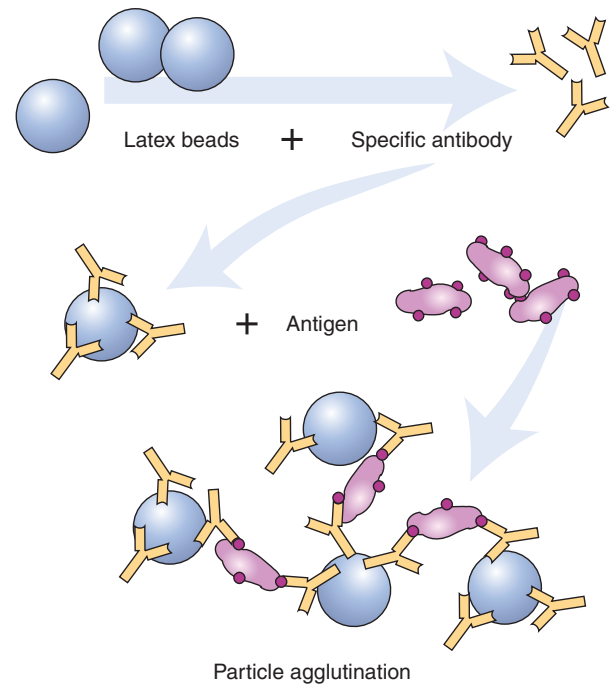


Fig. 10.6 Alignment of antibody molecules bound to the surface of a latex particle and latex agglutination reaction.

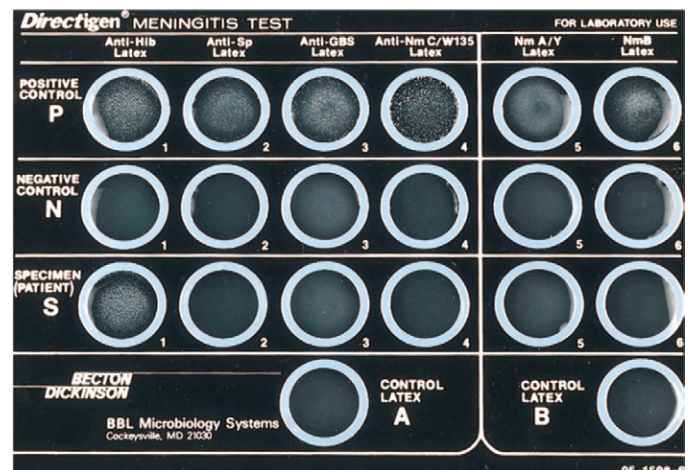


Fig. 10.7 Commercial latex agglutination test slide showing reaction of controls and a patient's cerebrospinal fluid with latex reagents specific for *Haemophilus influenzae* serotype b (Hib), *Streptococcus pneumoniae* (Sp), group B streptococcus (GBS), and five different serogroups (groups C and W135, groups A and Y, and group B) of *Neisseria meningitidis* (Nm). Note the positive reactions with each test latex reagent and positive control (P) as well as the positive reaction with the patient's specimen and Hib test latex. (Courtesy BD Diagnostic Systems, Sparks, MD.)

in the test sample, a maximum agglutination reaction (4+ on a 1+ to 4+ scale) may occur in only a few seconds. In the absence of a specific antigen, the latex suspension remains homogeneous and milky; that is, no agglutination is seen.

Controls must accompany the testing of patient specimens (Table 10.2). These controls include (1) a positive antigen control (a solution containing the known antigen of interest), (2) a negative antigen control (a solution not containing the antigen), and (3) a control latex suspension to detect the presence

Table 10.2 Interpretation of latex agglutination test controls and patient specimens

Reaction number	Test specimen	Agglutination reaction		
		Test latex	Control latex	Interpretation
1	Positive antigen control	+	0	Control okay
2	Negative antigen control	0	0	Control okay
3	Patient A	+	0	Positive test
4	Patient B	0	0	Negative test
5	Patient C	+	+	Nonspecific agglutination

+, Agglutination; 0, no agglutination.

of nonspecific agglutination reactions. The control latex involves testing the patient specimen with latex beads coated with an immunoglobulin whose specificity is not directed to the test antigen. This nonimmune serum is generally obtained from the same animal species in which the specific antibody was made. A nonspecific agglutination reaction occurs when the patient's specimen reacts with both the test and the control latex. When such reactions occur, the test is uninterpretable. A positive test result requires that the test latex, but not the control latex, agglutinate the patient specimen.

LA assays for microbial antigens, similar to other laboratory tests, are subject to false-negative and false-positive reactions compared with culture. False-negative reactions (negative antigen test result, positive culture) may be caused by the presence of antigen in the specimen at concentrations below the test detection limit. **Prozone** occurs when the relative concentration of antibody exceeds the antigen concentration. In this situation, each antigen combines with one or two antibody molecules, and cross-linking between the antigen and antibody does not occur. In addition, false-negative reactions can occur with antigen excess; this is called **postzone**. When the antigen concentration exceeds the relative antibody concentration, cross-linking between the antigen and antibody does not occur, and a proper lattice does not develop. Some test kits for antigen require all negative results be retested with a diluted sample to rule out postzone.

False-positive reactions (positive antigen test result, negative culture) are more difficult to explain; they may be caused by the presence of cross-reacting antigens or nonviable organisms in the specimen. Antigen detection tests do not require viable microbes. Antibody-coated latex suspensions can agglutinate viable or nonviable organisms, microbial components such as cell wall or membrane fragments, or soluble antigens such as bacterial capsular polysaccharide. Thus an apparent false-positive latex test result may actually represent a true-positive result for disease in a patient with a negative culture.

Numerous specimen pretreatment procedures can be used to eliminate or minimize nonspecific agglutinations, presumably by removing or inactivating factors in the specimen

responsible for these reactions. These procedures include specimen centrifugation to remove particulate material, boiling to inactivate protein constituents (acceptable when test antigen is a heat-stable polysaccharide), and passing the specimen through a membrane filter. Some specimens, such as urine, may be concentrated by centrifugation or membrane filtration before testing. Filters typically trap or exclude high-molecular-weight antigenic materials while allowing water and small compounds to pass through. These concentration methods increase the sensitivity of the test. Pretreatments are less frequently required as commercial latex kits become more specific and sensitive.

The advantages of LA tests are the availability of good-quality reagents in complete kit form, good sensitivity, relative rapidity, and ease of performance. Disadvantages include subjectivity in reading endpoints and nonspecific reactions resulting from interfering substances in clinical samples. The LA test is one of the most widely used antigen detection methods in the clinical laboratory. In many clinical laboratories, serogroup identification of the β -hemolytic *Streptococcus* spp. from culture plates is routinely performed using LA test kits. The sensitivity for *Streptococcus pyogenes* in throat swabs is 70% to 96%. Commercial antibody-detection serologic tests based on LA are available for many viral infections, including CMV, rubella, and infectious mononucleosis (heterophile antibody), and a few bacterial (streptococcal, mycoplasmal), fungal (candidal), and parasitic (toxoplasma) infections. Because of low sensitivity, routine use of LA tests for the detection of *Streptococcus agalactiae* in CSF and urogenital tract specimens is no longer recommended.

Staphylococcal coagglutination

Similar to LA, staphylococcal coagglutination, or simply **coagglutination**, uses particle-bound antibodies to enhance the visibility of antigen-antibody reactions. Instead of a latex bead, intact formalin-killed *Staphylococcus aureus* cells (typically Cowan 1 strain) are used. The cell wall of this strain of *S. aureus* contains a large amount of protein A, which binds the Fc portion (crystalline fragment, back of the heavy immunoglobulin chains) of the IgG antibody molecule. This leaves the fragment of antigen-binding (fab) sites of the molecule available to react with specific antigens (Fig. 10.8). It has been estimated that each staphylococcal cell has about 80,000 antibody-binding sites. The actual number of antibody molecules bound to the staphylococcal cell is limited by steric hindrance. However, coating is sufficient to render the product of clinical utility in direct antigen tests.

Coagglutination reactions are prepared by mixing antibody-sensitized staphylococcal cells with a solution containing the antigen of interest on a slide or card (Fig. 10.9). Coagglutination procedures appear more susceptible to nonspecific agglutination reactions, and specimen preparation is important. This is particularly true for testing serum specimens, probably because staphylococcal cells may bind human IgG in test serum specimens and subsequently be agglutinated by the presence of RF (anti-IgG IgM) in the serum. Coagglutination is highly specific but may be less sensitive than LA in detecting small quantities of antigen. Therefore these reagents are often used to confirm the identification of bacterial colonies on culture plates but not for rapid antigen detection from clinical specimens.

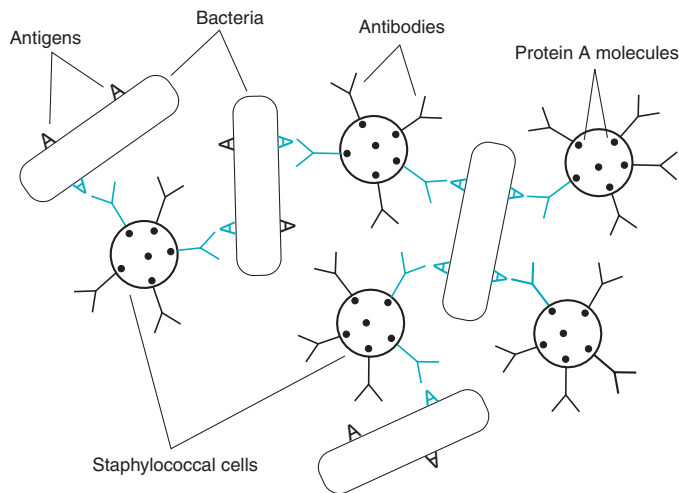


Fig. 10.8 Diagram of coagglutination reaction with whole bacterial cell antigen. A, Antigen.

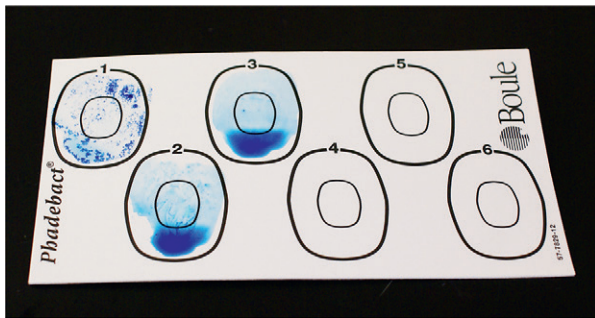


Fig. 10.9 Commercial coagglutination test card showing positive (1) and negative (2 and 3) reactions. The blue color is an indicator dye added to make the agglutination reaction easier to read against a white background.

Hemagglutination

In passive or indirect hemagglutination (IHA), microbial antigens are attached to erythrocytes after chemical treatment of the cells with tannic acid, chromic chloride, glutaraldehyde, or another substance that promotes cross-linking of the antigens. The sensitized cells can be reacted with a patient's serum specimen to detect agglutinating antibody. This assay method is rarely used today. Cells such as erythrocytes have a surface negative charge known as the **zeta potential**. If bacterial or fungal cells have the same charge, they will repel. For agglutination to occur, antibodies binding to the cells must be sufficiently large to span the cells and overcome the charges. IgM, because it is a pentamer, is better at agglutinating erythrocytes than IgG. The repulsion of cells by the zeta potential can be affected by pH, low ionic salt solution, and fluid viscosity.

An example of a direct hemagglutination test is the detection of heterophile antibodies in the acute stage of infectious mononucleosis, resulting from Epstein-Barr virus (EBV) infection. In the case of infectious mononucleosis, human heterophile antibody reacts with horse, ox, and sheep erythrocytes. In the appropriate setting, heterophile antibody testing is diagnostically useful. Specially treated horse erythrocytes provide the antigen, and the agglutination test is read on a slide or card (BBL MonoSlide; Beckton-Dickinson, Franklin Lakes, NJ). Another company markets dyed, color-enhanced



Fig. 10.10 Sure-Vue Mono test kit for the detection of heterophile antibodies produced during infectious mononucleosis. (Courtesy Biokit USA, Lexington, MA.)

horse erythrocytes for better contrast (ColorCard Mono-Test; Alere, Waltham, MA). Newer assays are based on heterophile antigens attached to latex particles (Sure-Vue; Biokit USA, Lexington, MA) (Fig. 10.10).

Hemagglutination inhibition

A laboratory test that has great historic significance to diagnostic serology, particularly to viral infections, is the hemagglutination inhibition (HI) test. This test takes advantage of the fact that many viral agents, including rubella and influenza viruses, have surface antigens that can agglutinate erythrocytes from certain mammalian species. Antibodies present in sera can bind to the virus and inhibit the agglutination reaction. The HI antibody titer is the highest dilution of the patient's serum that completely inhibits agglutination of the erythrocytes by the virus. Enzyme immunoassays (EIAs) are frequently used to diagnose influenza virus infection as well. These assays have the advantage of being able to differentiate IgG from IgM.

Liposome-mediated agglutination

Liposome-mediated agglutination involves the use of liposomes, which are single-lipid bilayer membranes that, under appropriate conditions, form closed vesicles ranging in size from 1 to 20 μm in diameter. In their manufacture, antigen or antibody molecules can be incorporated into the surface of the membrane and be available for interaction with the corresponding molecule (Fig. 10.11). In addition, the liposome vesicle may be constructed with a chemical dye or bioactive molecule trapped in the interior. The colored dye allows easy visual detection of lattice formation and agglutination between liposome-bound antibodies and antigens. Alternatively, combining dye-containing liposomes and latex beads, both of which contain the reactive antibody on the surface, may increase the sensitivity of LA. Another potential

advantage of liposome technology is in its application to immunoassays other than particle agglutination that make use of the ability of the liposome to carry reactive chemicals. Liposomes have yet to reach their full potential as diagnostic reagents in the clinical laboratory. Particle agglutination methods are compared in Table 10.3.

Neutralization assays

Neutralization assays for antibody detection are based on the interaction of a biologically active antigen with antibodies that can block or inactivate the activity of the antigen. Not only involved in serologic tests, neutralizing antibodies play an important role in functioning as protective antibodies *in vivo*. For example, immunity to numerous viruses depends on circulating antibodies that can neutralize, or block the infectivity of viruses that reach the bloodstream. Neutralization probably occurs because the antibody binds to the viral particle and blocks the interaction between viral adhesion molecules and receptor sites on target cells. Neutralizing antibodies to viruses can be measured *in vitro* using cell cultures.

In viral neutralization, a patient's serum sample is serially diluted, and each dilution is mixed with a standard amount of a known virus suspected of causing disease in the patient. The virus-serum mixtures are inoculated to a series of cell

culture tubes or flasks. If the patient's serum contains neutralizing antibodies to the virus, the antibody blocks viral infection and prevents the **cytopathic effect** (CPE) of the cells. CPE is the visual changes occurring in cell monolayers caused by viruses or toxins. The neutralizing antibody titer is the highest dilution of the patient's serum to completely block the CPE. For diagnosis of current disease, acute-phase and convalescent-phase sera should be tested, and a fourfold titer increase should be demonstrated.

Viral neutralization assays are highly sensitive and specific, but they also are technically demanding and require the use of active virus and cell cultures. They are not commonly used serologic tests today, but neutralization tests are valuable in identifying an unknown virus with known antibody. Other examples of neutralization assays are the HI assay (previously discussed) and the antistreptolysin O (ASO) neutralization test to detect antistreptococcal antibodies.

Labeled immunoassays

Immunofluorescent assays

Immunofluorescent assays are a method for rapid antigen and antibody detection in the clinical laboratory. Results are generally available within 15 to 60 minutes. When specific monoclonal or polyclonal antibodies are conjugated with fluorescent dyes (*fluorochromes*), they can be visualized with a fluorescence microscope. Labeled antibodies are called **conjugates** because the antibody is conjugated (linked) to a label. Fluorochromes are chemicals that absorb light of one wavelength and emit light of a different wavelength, thus changing color. The fluorescence microscope uses an epiluminescent (incident) light system of vertical illumination, wavelength filters, and a dichroic mirror (Fig. 10.12). The dichroic mirror allows passage of light at an excitation wavelength from the light source to the specimen. The mirror also allows passage of the emission (longer) wavelength light from the labeled source to the objective lens. This emitted, or excited, wavelength light appears as a bright color depending on the dye. A commonly used fluorochrome is fluorescein isothiocyanate (FITC), which, when excited, emits a bright apple-green fluorescence (Fig. 10.13) that can be seen against a black background.

Detection techniques may be direct or indirect. In the **direct fluorescent antibody** (DFA) test, the clinical specimen containing the antigen of interest is fixed onto a glass slide with formalin, methanol, ethanol, or acetone. Fixation renders mammalian cell membranes permeable to the stains. The antigen-specific labeled antibody is applied to the fixed

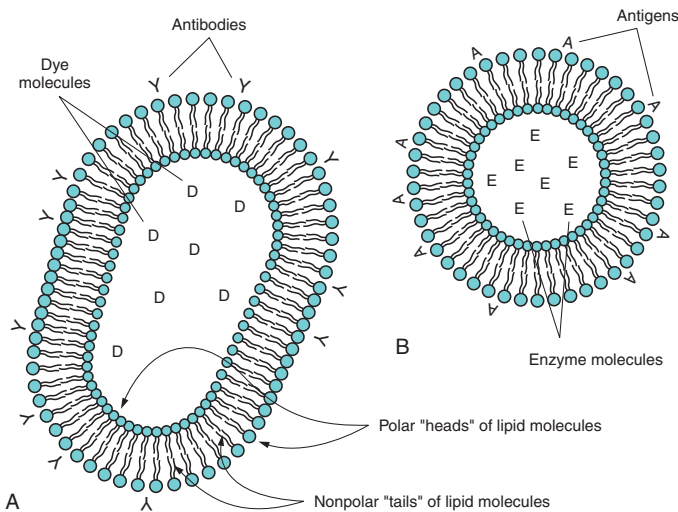


Fig. 10.11 Diagram of liposome particles showing bilipid layer structure. Either antibody (A) or antigen (B) can be attached to the surface of the liposome. The interior of the liposome can carry reporter molecules (e.g., dyes, enzymes).

Table 10.3 Comparison of particle agglutination methods			
Agglutination method	Particle type	Nature of antibody-binding to particle	Diagnostic utility
Latex agglutination	Latex beads	Nonspecific absorption or chemical coupling	Most widely used agglutination method for direct detection
Staphylococcal coagglutination	Formalin-fixed staphylococci	Fc portion of IgG molecule binds to protein A on staphylococcal cell wall	Used for antigenic identification of bacteria in culture
Liposome-mediated agglutination	Liposomes	Chemical coupling	Newer method; limited applications of commercial tests
IgG, Immunoglobulin G.			

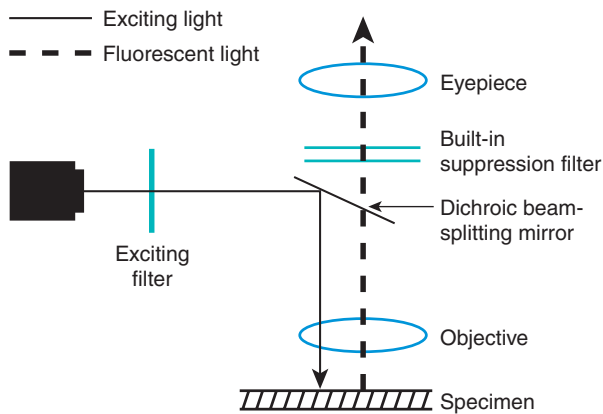


Fig. 10.12 Light path of incident light microscope.

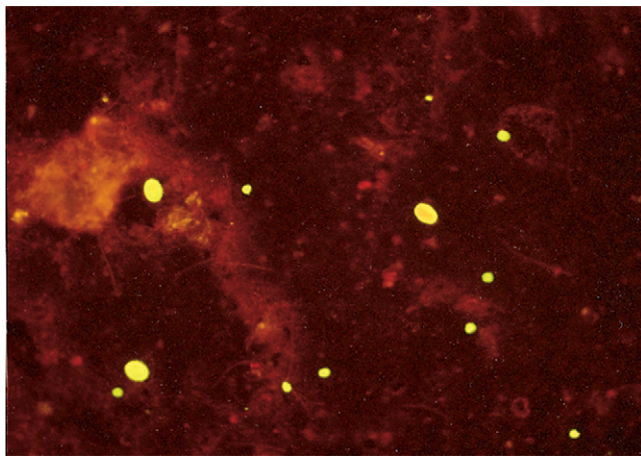


Fig. 10.13 Direct fluorescent antibody–stained cells of *Giardia duodenalis* (three larger apple-green, oval cells) and *Cryptosporidium* spp. (smaller cells) in stool. (Courtesy Meridian Bioscience, Cincinnati, OH.)

specimen, incubated, washed, and examined with a fluorescence microscope. If the antigen was present in the clinical specimen, the labeled antibody (conjugate) binds to the antigen, and fluorescence is seen (Fig. 10.14A). Immunofluorescent staining allows for rapid visualization of infected tissue, cell culture, body fluids, and swab specimens.

When the infectious agent does not produce enough antigen for detection in body fluids, direct testing of host cells must be considered. The use of a specific antibody for the infectious agent enables the detection of the agent inside the infected host cells. Direct immunofluorescence techniques are the assay of choice under these conditions. The DFA test is used to detect *Bordetella pertussis*; *Legionella pneumophila*; *Giardia duodenalis* (synonyms *G. lamblia*, *G. intestinalis*); *Cryptosporidium* spp.; *Pneumocystis jirovecii*; herpes simplex virus; CMV; varicella-zoster virus; and commonly isolated respiratory viruses such as parainfluenza virus, influenza virus, adenovirus, and respiratory syncytial virus (RSV) in clinical specimens. Immunofluorescent assays can also be used for confirmation of viruses isolated in cell cultures.

In the **indirect fluorescent antibody (IFA)** test for an antibody, whole microbial cells (bacteria, fungi, protozoan parasites) or virus-infected mammalian cells are washed and fixed

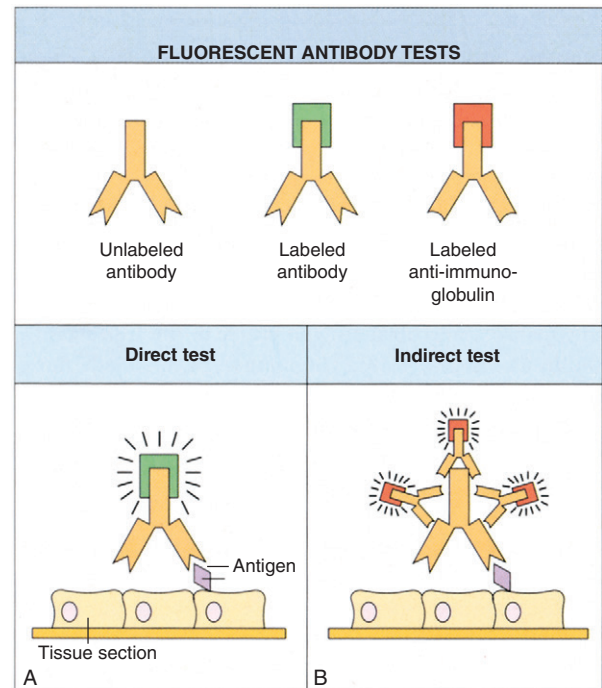


Fig. 10.14 Fluorescent antibody test for identification of tissue antigens or their antibodies. **A**, Direct fluorescent antibody. The antigen-specific–labeled antibody is applied to the fixed specimen, incubated, washed, and visualized with a fluorescence microscope. **B**, Indirect fluorescent antibody. A second fluorochrome-labeled antibody specific for the first unlabeled antibody is applied. (From Goering, R. V., et al. [2008]. *Mims' medical microbiology* [4th ed.]. London: Mosby.)

at a specific density to glass slides. Frequently with commercially prepared assays, cells are placed into small wells on the glass slide, and different patient samples are added to individual wells. Fixation is usually accomplished with methanol, ethanol, or acetone.

The patient's serum can be serially diluted, applied to the fixed-cell preparations, and incubated at 22° to 37°C in a humid environment to prevent drying, allowing antigen-antibody binding to occur. The slides are then washed with a buffered solution to remove the unbound antibody before application of antihuman IgG or IgM antibody that is fluorescently labeled (conjugate) and reactive with human immunoglobulin. The slides are incubated again and washed, air-dried, and viewed using a microscope fitted with a light source and filters to excite the fluorochrome (see Fig. 10.14B). If the specific antibody to the infectious agent's antigen is present in the patient's serum, the antibody binds, and secondary binding by the conjugate occurs. The cells fluoresce and are scored on a semiquantitative scale as positive (1+ to 4+) or negative. The titer is the reciprocal of the highest dilution of the patient's serum giving a minimum level of fluorescence (often at 2+ or greater).

The IFA test is also useful in detecting IgM-specific or IgG-specific antibody by using an FITC-labeled second antibody that is highly specific for human IgM or IgG. As with any IgM test, IFA for IgM is subject to false-negative results because of excess IgG and to false-positive results because of RF. To avoid these problems, IgG should be physically removed or functionally inactivated for IgM-specific assays.

The utility of DFA and IFA tests is limited by many factors, including the labor-intensive nature of the procedures, the requirement for a fluorescence microscope, the need for a darkroom, and the subjective interpretation involved in reading the slides. The procedures are not easily amenable to automation. Finally, fluorescence fades over time. The method is still sometimes used for the serologic diagnosis of infectious diseases caused by the TORCH agents, *L. pneumophila*, *B. burgdorferi*, *Rickettsia rickettsii*, and *M. pneumoniae*. The detection of *T. pallidum* antibodies using the fluorescent treponemal antibody absorption (FTA-ABS) test (discussed later in this chapter) is being replaced by assays that are easier to perform. The principle of IFA is also applied to the detection of autoantibodies (antinuclear antibodies) in the diagnosis of systemic lupus erythematosus and other autoimmune diseases.

Numerous variations of the IFA test have been developed. The basic objective in most of these procedures has been to increase sensitivity while maintaining specificity of the assay. One example of such a procedure is the double IFA test (Fig. 10.15). This method uses a second, unlabeled antihuman IgG or IgM antibody to bind to microbial-specific antibodies in a patient's serum. The FITC-labeled antibody is directed at the second antibody. The additional step amplifies the reaction because multiple molecules of the second antibody can bind to the patient's antibodies, providing additional binding sites for the FITC-labeled antibody. Newer serologic assays are often evaluated by comparison with IFA procedures. The IFA test is also quickly adaptable to studying serologic response to newly discovered infectious agents.

Enzyme immunoassays

Because of the drawbacks of immunofluorescent assays, antibodies have been conjugated to other labels. EIA is an alternative to immunofluorescent assays for detecting antigens and antibodies in clinical samples. Instead of labeling an antibody with a fluorochrome, EIA uses enzymes, such as horseradish peroxidase or alkaline phosphatase, conjugated to antibodies so both enzymatic and antigen-binding activities are preserved. When the appropriate substrate is added to the antigen-conjugated antibody complex, the enzyme catalyzes the production of a visible (colored) product that can be qualitative (positive or negative) or quantified spectrophotometrically. These products do not fade with storage and can be

detected by simple light microscopy. The ability to visualize and evaluate reactions gives a high level of certainty to the procedure.

EIAs are widely used for the serologic diagnosis of infectious diseases. They are popular for many reasons, including (1) the availability of commercial EIA kits for many infectious agents; (2) the adaptability of EIA tests to automation, allowing more tests to be performed in shorter times; and (3) the objective interpretation of test results with colored end products that can be read by a spectrophotometer. Several commercial products are tailored for high-volume laboratories; others are designed for single-use applications on individual patient specimens.

Commercially developed EIAs for the diagnosis of infectious agents typically require a solid phase for attachment of an antigen or antibody. Various solid matrix platforms are available that include individual wells of polystyrene microtiter plates (Fig. 10.16A), spherical plastic beads, or magnetic beads (see Fig. 10.16B). The solid matrix allows for washing of the sample and reagent to decrease nonspecific binding or background activity.

When performing an EIA to detect an antigen, the solid phase contains a capture antibody bound to the matrix. A clinical sample is added, and if the antigen of interest is present in the sample, it forms a stable immune complex with the antibody bound to the matrix. Unbound sample is removed by washing, and a second antibody specific for the antigen is added. In the direct method for antigen detection, this second antibody is conjugated to an enzyme. In the indirect method, a second nonconjugated antibody is added and washed, and then a third antibody specific for the second antibody is added. The third antibody is conjugated to the enzyme and is directed against the Fc portion of the unlabeled second antibody.

In either method, after the conjugate is added and washed, the enzyme-specific substrate is added. After a specified time, a stop reagent is added to block enzyme activity. The amount of colored product is directly proportional to the amount of enzyme-bound conjugate and antigen present in the original clinical sample. Both methods of detection are described in a step-by-step manner in Fig. 10.17. The methods described are often called **direct sandwich immunoassay** and **indirect sandwich immunoassay**. The advantage of the indirect sandwich immunoassay is the need for only one enzyme-conjugated anti-immunoglobulin antibody (third antibody) that can be used in different assays to detect various antigens. A disadvantage is that heterophile antibodies can produce false-positive or falsely increased results. The heterophile antibody can behave like the antigen, binding to both the capture and the signal antibodies.

In **competitive immunoassays**, an unlabeled patient antibody competes with a commercially prepared labeled antibody for a limited number of binding sites to an antigen. The clinical specimen and labeled antibody are added simultaneously, and after appropriate time, they reach equilibrium. The more antibodies present in the clinical sample, the fewer labeled antibody molecules bind. After a wash step, the enzyme substrate is added. The color produced is inversely proportional to the antibody concentration in the specimen. In other words, the greater the color intensity, the lower the patient antibody concentration. This method can also be applied to determining antigen

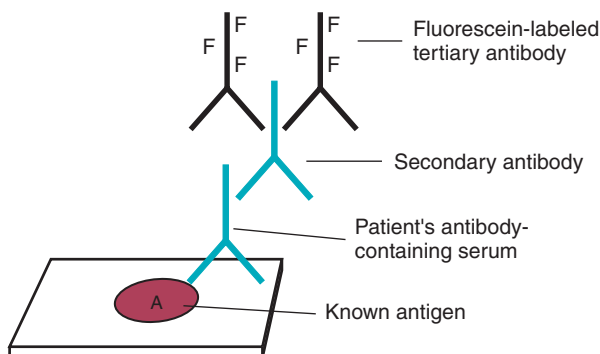


Fig. 10.15 Double indirect fluorescent antibody tests for antibody detection.

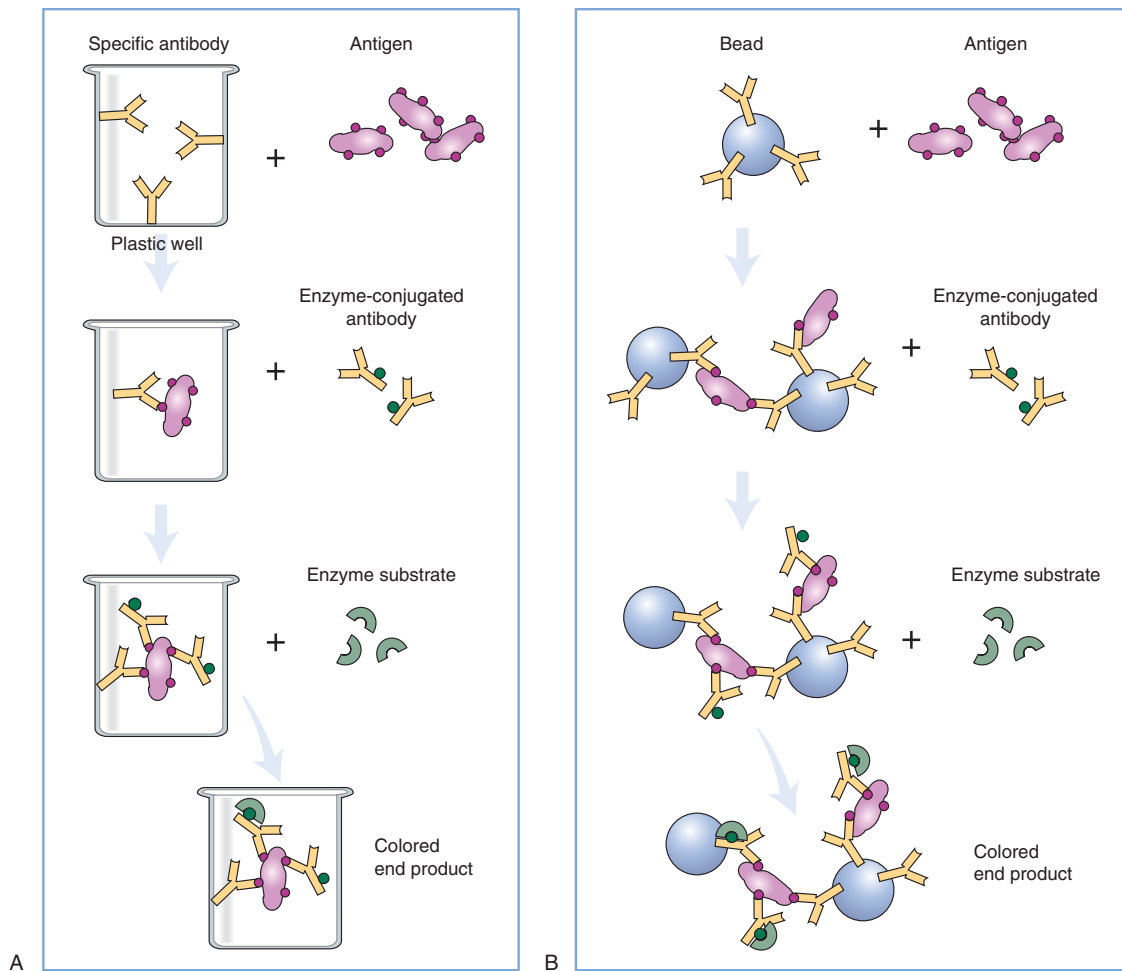


Fig. 10.16 Principle of direct solid-phase immunosorbent assay. **A**, Solid-phase surface is a microtiter well. **B**, Solid-phase surface is a bead.

(analyte) concentration. A capture antibody is attached to a solid phase, and a patient's sample containing unlabeled antigen is added with the labeled antigen. After incubation, the substrate is added, and again, the color intensity is inversely proportional to the antigen concentration. Competitive assays have greater sensitivity than noncompetitive assays and are frequently used to measure the concentration of analytes, such as hormones.

Commercial companies have produced good-quality EIA kits for detection of various microbial antigens. Conjugates can be inexpensively prepared, are stable for 6 months, and have reasonably good sensitivity in most applications. The use of microtiter plates and instrumentation for dispensing reagents, wash procedures, and automated optical reading of reactions greatly facilitated EIA methods to allow large volumes to be batch tested (Fig. 10.18). However, some methods are time-consuming and may not be practical for low-volume laboratories or "stat" runs. They cannot always be considered rapid tests compared with other procedures such as LA. EIAs are used for rapid detection of *L. pneumophila*, *Clostridioides difficile* toxins, *G. duodenalis*, and *Cryptosporidium* spp. Because of low sensitivity, EIAs for *Chlamydia trachomatis* are considered substandard and are no longer recommended. Nucleic acid amplification tests (NAATs) are regarded as the tests of choice in clinical specimens.

Immunoassays for the detection of serum antibodies are performed in a manner similar to immunoassays for microbial antigen detection, except that the roles of antigen and antibody are reversed (Fig. 10.19). Most serum antibody assays are performed on a solid matrix using antigen-coated tubes or wells. Because of the large number of tests that can be performed and the small serum volume required, 96-well microtiter plates are commonly used for this purpose. Single-test, single-serum dilution cassettes are also available from commercial sources for settings with low-volume or infrequent testing. In either case, the patient antibody binds to the microbial antigen-coated surface, and the antibody is detected using an enzyme-labeled antihuman immunoglobulin, or conjugate. EIAs or, more specifically, solid-phase EIAs (enzyme-linked immunosorbent assay [ELISA]) are the most popular types of immunoassay in use today.

Many laboratories use some form of ELISA testing for antibody detection. In this test, results are reported not as antibody titer, but as the results relate to the relative amount of signal generated by the patient's serum compared with that of a known, weakly positive specimen. Results are usually reported as either relative units with a numeric reference range (U/mL) or as a ratio of results in the sample to the low positive control (e.g., patient 15 U/mL, control 10 U/mL, ratio 1.5). If the test result is above an established threshold,

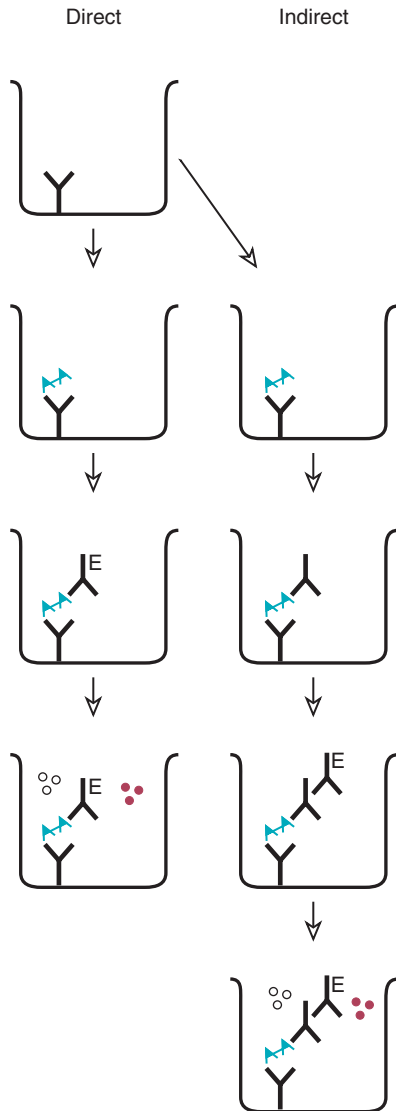


Fig. 10.17 Principle of various enzyme immunoassays for antigen. Both methods (direct and indirect sandwich immunoassays) start with specific antibody bound to the solid phase. Arrows separate steps in the procedures where washing of the solid phase takes place. Y, Antibody; A-A, antigen; E, enzyme; enzyme substrate, enzyme product.

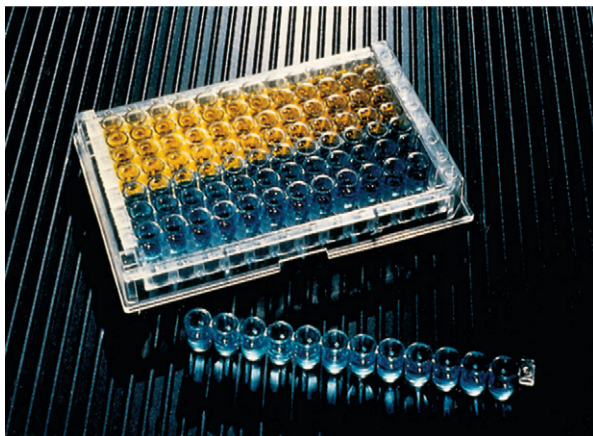


Fig. 10.18 Enzyme immunoassay test in microtiter plate (top) and strip (bottom) showing wells with product of enzymatic reaction before (blue) and after (yellow) addition of an acidic stop solution. (Courtesy Meridian Bioscience, Cincinnati, OH.)

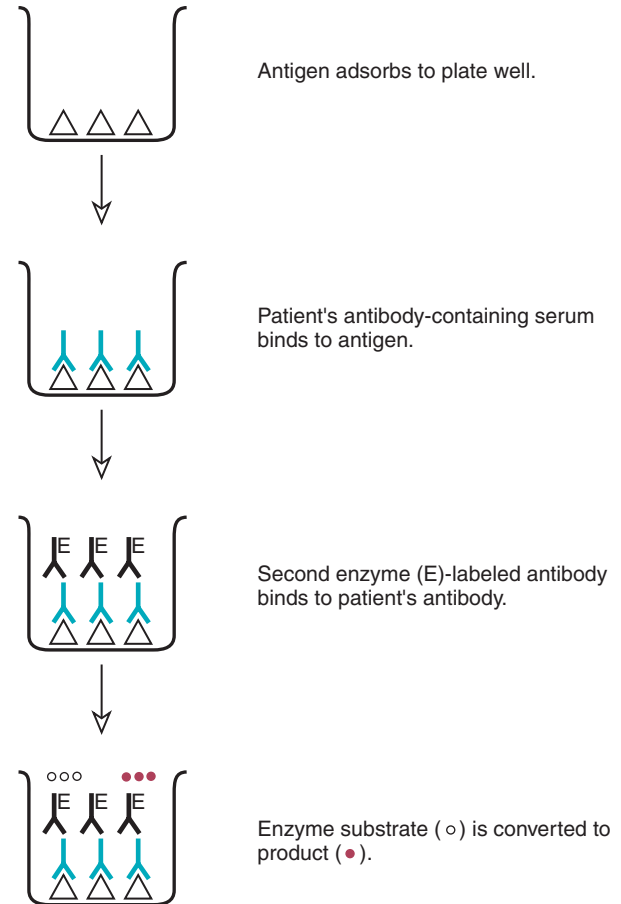


Fig. 10.19 Principle of indirect solid-phase enzyme immunoassay (EIA) for antibody detection.

the result is considered positive or reactive. It is impossible to compare the results of serial dilution methods with ELISA results because of the variation from test to test. However, there is a linear relationship in each individual assay between the amounts of antibody present, which means that a doubling in the EIA result yields a twofold increase in the antibody titer.

The *IgM antibody capture* ELISA specifically detects IgM antibody (Fig. 10.20). In this procedure, the solid phase is first coated with an animal antibody specific for human IgM. The patient's serum is added and incubated, and the tube or plate is washed. At this point, IgM antibody molecules, regardless of antigen specificity, should be bound to the solid phase, and antibodies of other immunoglobulin classes should be washed away. The next step in the assay involves adding the antigen of interest and can be performed in one of two ways. The antigen can be directly labeled with an enzyme or can be added unlabeled. In either case, the antigen will bind to the solid phase if the specific IgM antibody was present. With labeled antigen, the assay is completed by adding enzyme substrate and measuring the colored product. Alternatively, unlabeled antigen bound to IgM is detected by adding a secondary enzyme-labeled antibody directed against the specific antigen, followed by the addition of enzyme substrate. The amount of colored product is directly proportional to the amount of specific IgM captured on the solid phase.

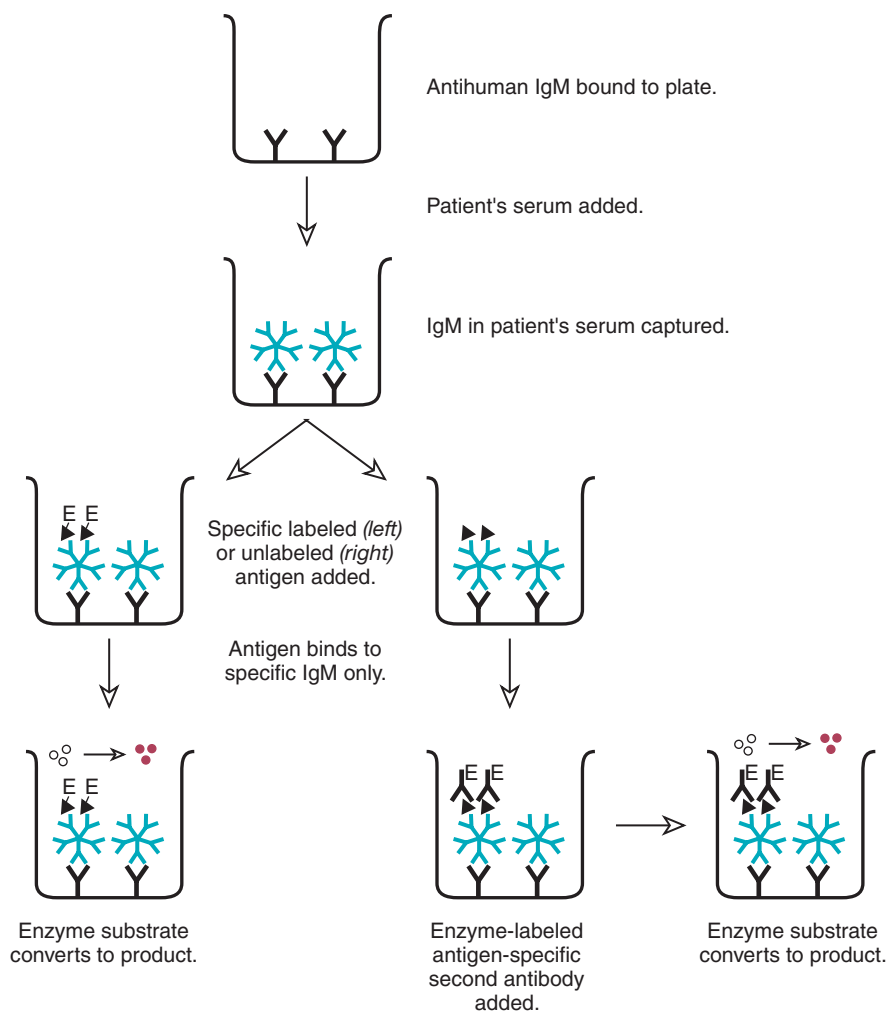


Fig. 10.20 Immunoglobulin M (IgM) antibody capture enzyme-linked immunosorbent assay using labeled antigen (*left*) or unlabeled antigen; a secondary enzyme-labeled antibody is added later (*right*).

The IgM antibody capture assay has the advantage of eliminating the need for separation of IgG that might compete with IgM and yield false-negative results. The capture assay may be susceptible to false-positive results because of IgM RF bound to the solid phase. Modifications of the assay can be employed to reduce the possibility of this problem. Another way to distinguish between IgG and IgM is to use IgG-specific and IgM-specific conjugates.

A highly sensitive EIA variation utilizes **biotin-streptavidin systems**. Streptavidin can be linked to enzymes, often horseradish peroxidase, or fluorochromes such as fluorescein or rhodamine. Streptavidin is preferred over avidin because of greater sensitivity and a lower incidence of nonspecific binding. It binds very tightly and specifically to biotin by one of four reactive sites on each streptavidin molecule. When detecting an antigen in a clinical specimen, the antibody directed against the antigen is biotinylated. A specimen from the patient is fixed to a solid surface, and the biotinylated antibody is added. Following a wash step, streptavidin conjugated to an enzyme is added. After another wash step, the enzyme substrate is added, and a colored product is formed. The biotin-streptavidin interaction takes the place of the primary antibody–secondary antibody interaction. This assay

can be adapted for antibody detection by having the antigen attached to a solid surface, then adding patient sera. After washing, biotinylated antihuman antibody is added. This is followed by the addition of enzyme-labeled streptavidin as before. After this, the substrate is added. The biotin-streptavidin system increases the amount of signal detected (increased sensitivity) for the following reasons: (1) multiple biotin molecules can bind to the primary antibody and (2) each streptavidin molecule has four reactive sites for biotin. Biotin-streptavidin can also be used in competitive immunoassays.

Biotin-streptavidin assays have become very popular. A survey conducted in 2016 found that 59% of immunoassays (221/374) utilized this detection system. Because biotin (vitamin B7) is an essential nutrient, there are concerns that biotin in clinical specimens might interfere with these assays. Biotin in clinical samples could block the binding of biotinylated antibodies to the biotin binding sites on the streptavidin, resulting in falsely decreased results. Not all assays are equally affected by biotin, and it is likely that interference will only occur in patients taking biotin supplements. In these patients, laboratories should absorb biotin from clinical specimens with a pretreatment step using streptavidin microparticles.

Membrane-bound immunoassays

The flow-through and large surface area characteristics of nitrocellulose, nylon, and other membranes enhance the speed and sensitivity of EIA reactions. The improvements associated with membrane-bound EIAs are largely the result of immobilizing the antibody onto the surface of porous membranes. These assays use a disposable plastic cassette consisting of the antibody-bound membrane and a small chamber to which the antigen-containing clinical sample is added (Fig. 10.21). An absorbent material is placed below the membrane to pull, or wick, the liquid reactants through the membrane; this helps separate nonreacted components from the antigen-antibody



Fig. 10.21 TestPack Strep A kit components of membrane-bound enzyme immunoassay test for group A streptococcal polysaccharide antigen. (Courtesy Inverness Medical Professional Diagnostics–BioStar, Louisville, CO.)

complexes of interest that are bound to the membrane and simplifies the washing steps. Incubation times are also decreased because the rate of antigen binding is proportional to its concentration in solution near the membrane surface; this action increases the rate and extent of binding. Membrane-bound EIA tests are commonly used for the rapid antigen detection of rotavirus, RSV, influenza A and B viruses, *C. difficile* toxins, and group A streptococci (Fig. 10.22).

Lateral flow immunoassays, also called *immunochromatographic tests* (ICTs), are based on a similar principle. This assay can be used to detect an antibody or antigen. To detect an antigen in a clinical specimen, a capture antibody with specificity for the antigen in question is applied in a line (called the *sample* or *test line*) onto the membrane. Beyond the test line, a line of antirabbit antibody (control line) is attached. Below the test line on the membrane is rabbit antibody with specificity for the antigen in question labeled with a dye or colloidal gold. The clinical specimen is placed in a buffer diluent, and the processed specimen is added to the membrane. The buffer is carried laterally along the membrane by capillary action. The labeled rabbit antibody becomes suspended in the buffer, and if antigen is present in the clinical specimen, it forms a complex with the labeled antibody.

As the buffer migrates carrying the reactants, the antigen-antibody complex binds to the capture antibody affixed to the membrane at the test line. Enough of the complex binds to form a visible color, from the dye, on the test line. The control line is an internal control that must be positive for results to be valid. The control line captures any labeled rabbit antibody and is positive in specimens with or without the antigen. The control line ensures that the buffer has migrated past the test line and confirms a negative test result. This is the method used in home test kits for detecting severe acute respiratory syndrome coronavirus 2 antigen.

In an ICT assay to detect an antibody, such as anti-HIV (OraQuick; OraSure Technologies, Bethlehem, PA),

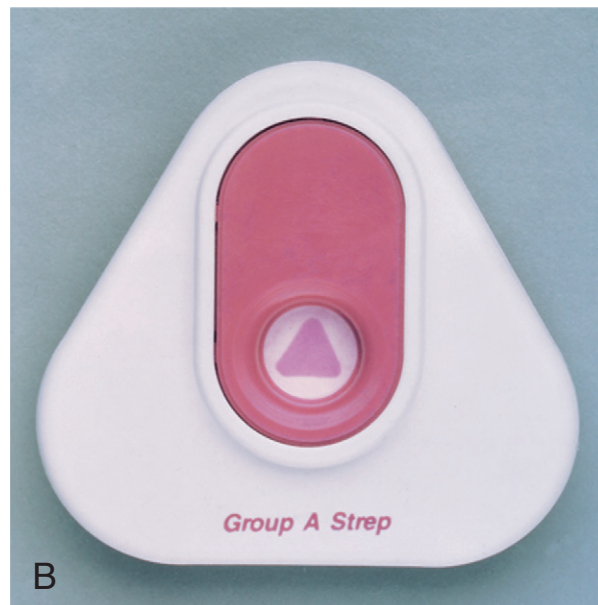


Fig. 10.22 Color PAC devices showing negative (A) and positive (B) reactions of liposome-enhanced membrane-bound enzyme immunoassay for group A streptococcal polysaccharide antigen. (Courtesy Becton, Dickinson and Company, Sparks, MD.)

staphylococcal protein A labeled with colloidal gold in the kit binds to the Fc portion of IgG with any specificity in the clinical specimen. The complex is carried to the test line, which contains HIV antigens. If anti-HIV antibody is present in the clinical specimen, it binds to the antigens and a red line develops. The control line contains antihuman antibody that binds IgG molecules not specific for the HIV antigen present in the specimen, producing a red line (Fig. 10.23). Because of their ease of use and built-in control, ICT assays have become popular in physicians' offices. Kits are available to detect numerous substances, such as drugs, and are used as field tests in forensic science.

Optical immunoassays

Optical immunoassay (OIA) relies on the interaction of antigen-antibody complexes on inert surfaces. Specific antigen-antibody



Fig. 10.23 Immunochromatographic test. The OraQuick test is a rapid assay for the detection of antibody to human immunodeficiency virus types 1 and 2. Formation of a red line in the test area is a positive reaction.

interaction alters the thickness of the reactants on the test surface. Light reflecting off the surface film containing an antibody only is viewed as one color. However, when a specific antigen is bound to an antibody, it increases the thickness of the film. This increased thickness causes the surface to appear a different color to the naked eye. An OIA is available to detect group A streptococci in pediatric populations (Fig. 10.24). An OIA also has U.S. Food and Drug Administration (FDA) approval for the detection of influenza A virus. A point-of-care test for *Neisseria gonorrhoeae* (BioStar; Alere) reacted positively with 99.4% of *N. gonorrhoeae* isolates and was negative with 88.7% of nongonococcal *Neisseria* isolates.

Complement fixation test

CF tests are versatile assays that can be used for both antibody and antigen detection. They are broadly applicable for various infectious agents and have been used for many years, particularly for antibody detection. In clinical laboratories, CF tests have been largely replaced by more rapid and sensitive assays; however, they are still used in reference and public health laboratories for certain agents.

Although the serum complement protein system plays a vital role in many immunologic functions, it is beyond the scope of this chapter (see Chapter 2). The proteins involved in the complement system are usually found in an inactive form; however, once activated, the proteins become involved in an enzymatic cascade that involves production of proteins with various biological activities. Activation of complement occurs by the classic pathway when specific IgG or IgM antibody combines with antigen and exposes a complement-binding site on the antibody molecule. It is the cell-lysing ability of activated complement components that is important in CF tests.

The CF test requires both an indicator system and a test system (Fig. 10.25). The indicator system typically consists of a combination of sheep erythrocytes, rabbit antibody to sheep erythrocytes, and guinea pig complement. When these three components are present and active, the rabbit antibody first combines with the sheep erythrocytes, and complement is subsequently bound (fixed) to the antibody-antigen complex, resulting in activation of complement by the classic pathway;

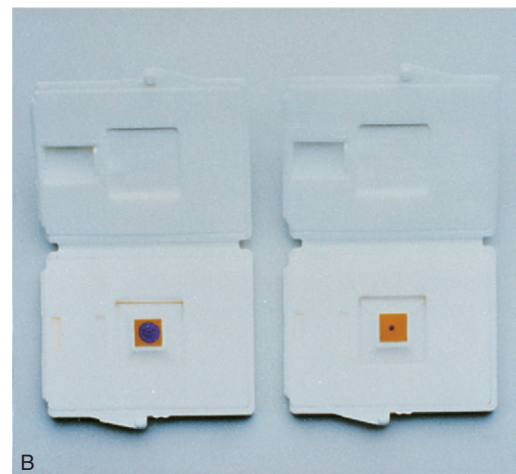
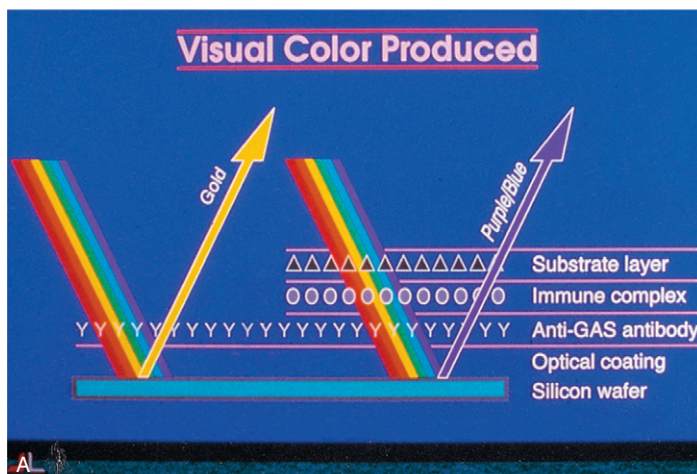


Fig. 10.24 Optical immunoassay for group A streptococcus (GAS). **A**, Principle of Biostar optical immunoassay for group A streptococcus showing color change from gold to purple after attachment of immune complex—containing group A streptococcal antigen. **B**, Test cassettes showing a positive reaction (left) and negative reaction (right). (Courtesy Inverness Medical Professional Diagnostics—BioStar, Louisville, CO.)

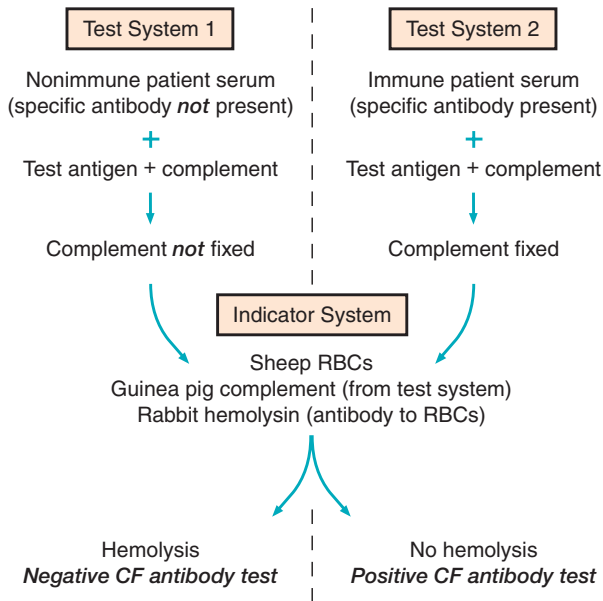


Fig. 10.25 Principles of complement fixation (CF) test. RBCs, Red blood cells.

this results in lysis of the sheep erythrocytes. The test system consists of a mixture of known antigen and a patient's heat-inactivated serum that may or may not contain specific antibody to the antigen. Heating the patient sample is necessary to inactivate complement present in human sera.

The CF test is performed as a two-step procedure. The patient's serum is serially diluted in test tubes to determine a titer, and each dilution is mixed with a known amount of antigen (test system). A fixed amount of complement is added to each tube, and the mixture is incubated. Next, the sheep erythrocytes and rabbit antibody are added (indicator system) to each tube, and the tubes are incubated again. If the patient's serum had specific antibodies to the test antigen, complement would be fixed by the test system and would be unavailable to lyse the erythrocytes. Therefore no hemolysis is a positive result for the presence of an antigen-specific antibody. If the patient's serum did not have the specific antibody to the test antigen, complement would be fixed by the indicator system, resulting in erythrocyte lysis. The CF antibody titer is the highest dilution of patient serum resulting in 100% hemolysis. It is usually read spectrophotometrically.

Western blot

Although serologic test methods for detecting an antibody such as IFA assays and EIAs provide excellent sensitivity and specificity in most clinical applications, they are limited in their ability to resolve the complex antibody response occurring during many infectious diseases. Because most antigens used in these assays are crude microbial and viral extracts, a positive result may represent an antibody response to one or many antigens. The EIA test can be designed to detect antibody to numerous individual antigens, but this requires the expensive and labor-intensive process of purifying antigens and running multiple EIAs. As an alternative, the technique of Western blotting is used. This technique allows for characterization of multiple antibodies with different idiotypes

to an infectious agent by electrophoretic separation of the microbial or viral antigens and then transfer to a nitrocellulose membrane.

Western blotting was previously used to confirm antibodies to HIV type 1 (HIV-1) in patients whose sera had been repeatedly reactive in EIA tests. However, it has been shown that using Western blotting for confirmation of reactive initial immunoassay results can produce false-negative or indeterminate results early during HIV infection. In addition, because of cross-reactivity, false-positive results were reported in 46% to 85% of patients with HIV-2 infection. Western blotting is still considered a confirmation test performed only after a positive screening test for the diagnosis of Lyme disease.

In the Western blotting procedure, a crude antigen preparation (e.g., a partially purified virus preparation) is heated with a detergent such as sodium dodecyl sulfate (SDS). The SDS releases individual polypeptides from complex proteins. The polypeptides are separated according to their molecular weight by polyacrylamide gel electrophoresis with SDS (SDS-PAGE). The separated polypeptides form invisible protein "bands" in the gel. The protein bands are transferred to an inert filter membrane support, usually made of nitrocellulose. The nitrocellulose membrane is a solid matrix to which the separated protein antigens tightly adhere and on which an antibody-antigen reaction can occur and be visualized—similar to a microtitration plate well with an ELISA.

Many commercial kits for Western blotting contain nitrocellulose membrane strips on which the protein antigens of interest have already been electrophoretically separated. For analysis, the nitrocellulose membrane is immersed in a patient's serum sample (usually diluted) and allowed to incubate. Specific antibodies in the sample, if present, bind to unique epitopes in the protein bands. After incubation, the nitrocellulose membrane is thoroughly washed to remove unbound antibody, and a labeled antihuman immunoglobulin (secondary antibody) binds to the primary antibody on the membrane. The secondary antibody is commonly labeled with an enzyme or biotin. The visualization of an antigen-antibody reaction at any protein band is accomplished by the addition of enzyme substrate (or enzyme-labeled streptavidin followed by enzyme substrate) and a final wash (Fig. 10.26).

Western blotting has added a dimension of versatility and specificity to immunoassays. It has helped resolve false-positive EIA results caused by antibodies cross-reacting with other infectious agents, autoimmune disorders, and technical error. It has also helped dissect and analyze the antibody response to individual proteins in complex mixtures. Western blotting is a procedure that must be well controlled and interpreted using strict criteria. Antibodies detected by other methods may not be detected by Western blotting techniques because the polypeptide antigens used are not native, intact proteins (SDS treatment denatures the proteins), and some antigens may not be transferred to the membranes. Western blotting is a qualitative assay; antibody titers cannot be determined. Despite these drawbacks, Western blotting is expected to continue to play an important role in the immunoserology of select infectious agents.

Dot blot

Dot blots are like Western blots, except the protein antigens are not electrophoretically separated and transferred to a solid surface. Instead, the proteins are purified and directly applied



Fig. 10.26 Western blot for detection of antibody to HIV-1. Strips 1 and 4 are high-positive strips; strips 2 and 5 are low-positive; strips 3 and 6 are negative controls. Strips 7 to 13 and strip 16 are positive reactions of patient sera. Strips 14 and 15 are negative reactions of patient sera. (Courtesy bioMérieux, Durham, NC.)

(blotted) to specific locations on the solid surface. An example is the ImmunoDOT *Borrelia* for Lyme disease (GenBio, San Diego, CA). Whole borrelial antigen, used to screen for antibodies, and four purified or recombinant proteins (for specificity) are applied to a nitrocellulose filter strip along with a positive control. Patient serum is added to the strip, and if an antibody to any of the proteins is present, they bind. An enzyme-labeled antihuman antibody is added, followed by the enzyme's substrate to detect binding of the patient's antibody. The strips are read manually.

Other labeled immunoassays

Besides enzymes and fluorochromes, other labels can be used to detect in vitro antigen-antibody reactions, including **radioimmunoassay** (RIA) and **chemiluminescent immunoassay** (CLIA). The principles are like EIA except a label other than an enzyme is used. Radionucleotides (usually iodine 125 or carbon 14) are substituted for enzymes in RIA. Although RIA was once the key method for antigen detection of numerous infectious agents, especially HBV, it has largely been replaced by EIA, which does not require use of radioactive substances. The CLIA uses chemicals, such as luminol, acridium esters, and ruthenium derivatives, that emit light. A luminometer is required to read CLIAs.

Use of serologic testing in specific diseases

As the sensitivity, specificity, and ease of use improved, serologic tests became a mainstay in clinical laboratories. Manufacturers produce kits for screening large numbers of samples and kits for confirming positive or negative screening results. Screening tests are designed to detect many

individuals who have a particular disease. However, because of the high sensitivity, these tests are prone to false-positive results. Remember, as sensitivity increases (more true positives), specificity decreases (more false positives). Positive screening tests should be confirmed with a more specific confirmatory test. In addition, some kits are for single use and designed for low test volume, whereas other kits lend themselves to large-volume testing and can be automated. See [Table 10.1](#) for lists of commonly used and commercially available serologic tests to detect antibodies and the organisms identified. This section discusses important diseases diagnosed by antibody detection. The next section examines direct antigen detection in the diagnosis of infectious diseases.

Serologic testing for syphilis

Sexually transmitted diseases (STDs) are among the most common infectious diseases in the United States. The most common of these diseases are genital warts, chlamydia, and genital herpes; however, syphilis is one of the most dangerous if left untreated. *T. pallidum* subsp. *pallidum*, the spirochete that causes syphilis, can be identified from a lesion or chancre. However, serologic testing for syphilis is the most common method of diagnosis. Two forms of testing exist for syphilis: screening tests and confirmatory tests. Screening tests generally do not use bacterial antigens and are referred to as *nontreponemal tests*.

The confirmatory tests use treponemal antigens and are very sensitive and specific, but they are not suited for testing large numbers of individuals (screening) because they are technically demanding and time-consuming, and like other diagnostic tests, they would result in decreased diagnostic accuracy if applied to a large population with a disease of low prevalence. Approximately 1% of the population would give a false-positive result. Confirmatory tests also cannot be used to monitor therapy. The four FDA-approved treponemal assays are the FTA-ABS test, the Serodia TP-PA (*T. pallidum* particle agglutination) test (Fujirebio America, Fairfield, NJ), the *T. pallidum* hemagglutination (TPHA) assay, and the microhemagglutination-*T. pallidum* (MHA-TP) test. In the TPHA and MHA-TP tests, specific treponemal antibodies are detected using treponemal antigen-coated erythrocytes (fowl for TPHA and sheep for MHA-TP). Because of ease of use, less technical time, and lack of an absorption step and fluorescent microscope, the TP-PA test is the most used confirmatory assay.

Nontreponemal antigen tests are technically easier and more rapid to perform, and they are the tests of choice for syphilis screening. The most used nontreponemal tests today are the Venereal Disease Research Laboratory (VDRL) and the rapid plasma reagin (RPR) tests. The third FDA-approved assay is the toluidine red unheated serum test (TRUST). The VDRL test, named after the laboratory that developed it, is a microscopic flocculation test using heat-inactivated serum and performed on glass slides. The test detects reaginic antibodies (reagin) that bind to an alcoholic solution of precisely defined cardiolipin-lecithin-cholesterol particles. During the infection, these compounds are released from damaged host cells, resulting in antibodies being directed against them. Because nontreponemal antigens are used, the test is less specific than those assays using treponemal antigens. False positives have been associated with pregnancy,



Fig. 10.27 Rapid plasma reagin (RPR) card test for detection of nontreponemal antibodies. (Courtesy Baxter U.S. Distribution, Division of Baxter Healthcare, Deerfield, IL.)

intravenous drug use, and diseases such as systemic lupus erythematosus, mononucleosis, malaria, tuberculosis, and AIDS.

The RPR test uses the same antigen as the VDRL test; however, carbon particles have been added so the flocculation reaction can be seen more clearly. The RPR assay can be performed on unheated serum or plasma and is a flocculation reaction read macroscopically (Fig. 10.27). The TRUST resembles the RPR assay, except the dye toluidine red is substituted for charcoal. The VDRL and RPR tests and the TRUST detect both IgM and IgG and may be performed as both qualitative and semiquantitative tests. Because of the extreme attention to reagent preparation and quality control required and the difficulty in reading results, the VDRL test has been replaced by the RPR test in most clinical laboratories. However, the VDRL assay can be used to test CSF for diagnosing neurosyphilis and to follow antibody titers in a newborn to diagnose congenital syphilis. The RPR test can be used on CSF as well. However, its sensitivity is only 58% compared with 67% for the VDRL test.

Serologic testing for streptococcal infections

S. pyogenes causes many common, acute, pyogenic (associated with pus or neutrophil response) infections, including pharyngitis and skin infections. In addition, the organism is responsible for certain nonsuppurative (nonpyogenic) diseases, such as acute rheumatic fever and poststreptococcal glomerulonephritis, which occur weeks after the acute infectious process and are thought to be autoimmune-mediated diseases; that is, the damage is caused by the host's immune response. Although pyogenic infections are best diagnosed by isolation of the organism in culture, nonsuppurative diseases occur at a time when the organism may no longer be present; thus serologic diagnosis is often performed. In addition, it may be necessary to diagnose infections by serology after antimicrobial therapy has been initiated.

The ASO antibody test is used to demonstrate serologic response to *S. pyogenes*. One method to detect ASO is to measure the ability of the patient's serum to neutralize the hemolytic ability of the streptococcal enzyme, streptolysin O. The patient's serum is mixed with a standard concentration of streptolysin O. If antibody to streptolysin O is present, it binds to the hemolysin neutralizing it. When red blood cells

(RBCs) are added, the RBCs are not lysed. If antibody to streptolysin O is not present in the serum sample, the hemolysin is not neutralized, and the RBCs are lysed.

Antibody titers are based on comparison of the patient sample with known standards. Antibody titers are reported as Todd units, which are named after E. W. Todd, who discovered streptolysins. A fourfold increase in titer is considered diagnostic for a previous *S. pyogenes* infection. Titers peak 3 to 6 weeks after infection. High titers of ASO antibody develop in most cases of upper respiratory tract infection and rheumatic fever, but the titers and percentages of patients with streptococcal wound infections and poststreptococcal glomerulonephritis who develop ASO antibodies are significantly lower. Serologic assays to additional streptococcal antigens are discussed in Chapter 15.

Serologic diagnosis of viral diseases

Rubella

Rubella is normally insignificant, except in women who are pregnant. This disease may cause a miscarriage, or it may cause congenital heart disease, cataracts, deafness, or brain damage in the fetus. It is extremely important to determine whether women who may become pregnant have immunity to rubella. Serologic testing is generally by EIA for either IgM or IgG antirubella antibodies. Women who are not immune or have a low antibody titer should be vaccinated before becoming pregnant. Rubella vaccine is part of the trivalent measles, mumps, and rubella vaccine that most children in developed countries receive.

Infectious mononucleosis

The heterophile antibody titer is one of the tests used to diagnose infectious mononucleosis caused by EBV. The term *heterophile antibody* refers to an antibody with an affinity to an antigen from more than one group or species. Humans rarely have antibodies to sheep RBCs. However, many patients with infectious mononucleosis have been shown to develop antibodies that agglutinate sheep, bovine, and horse RBCs. Commercial diagnostic kits are available to help diagnose infectious mononucleosis. These kits are generally known as *spot tests*, and some use a saline suspension of antigen derived from the RBCs of horses (e.g., BBL MonoSlide). If the patient has infectious mononucleosis, mixing of the test reagent with a drop of the patient's serum causes hemagglutination. Some diagnostic kits place EBV antigens on carrier particles—RBCs or latex beads. Either IgM or IgG antibodies can be identified by these methods. These tests are rapid and sensitive for screening; however, they do not positively identify EBV infection. Because EBV antigens are not used, other factors may increase heterophile antibody titers; therefore the test is not specific. Other tests, such as antibodies to the viral capsid antigen and the Epstein-Barr nuclear antigen, are often needed to confirm active infectious mononucleosis.

Hepatitis

Hepatitis refers to inflammation of the liver. Many chemicals and infectious agents can cause hepatitis; however, most

cases of hepatitis have a viral etiology. Whereas several different viruses have been linked to hepatitis, hepatitis A virus (HAV), HBV, and HCV are the most common causes. Because of the extreme difficulty of isolating viruses causing hepatitis, serologic assays are very important for the diagnosis of viral hepatitis.

Numerous HBV serologic markers, both antibodies and antigens, are present in patients' sera soon after onset of infection. Serologic assays are often performed as a profile or panel of tests. One of the earliest markers detected in infections is the hepatitis B surface antigen (HBsAg). Although the virus has numerous antigens, HBsAg is highly antigenic and easy to detect. Additional antigen assays include detection of hepatitis B core antigen (HBcAg) and hepatitis B e antigen (HBeAg). The detection of HBsAg in serum signifies that the patient is either ill with the disease or is a carrier and is potentially infectious. The laboratory can also test for antibodies to the various antigens: anti-HBsAg antibody, anti-HBcAg antibody, and anti-HBeAg antibody. The presence of anti-HBsAg indicates immunity. A more thorough discussion of HBV serology is found in Chapter 29. HBV is spread in human blood, and blood units are screened for this virus to reduce the risk of posttransfusion hepatitis.

The tests most often used for the diagnosis of HAV infection are anti-HAV IgM and anti-HAV IgG. A positive anti-HAV IgM test is suggestive of acute infection, whereas a positive anti-HAV IgG test is indicative of past exposure. In the 1980s, a gene associated with an HCV protein was cloned. This protein was used to develop a test to detect antibodies to HCV in the blood. This was the first time a viral genome was used to develop a serologic test without first isolating the causative agent. Research determined that the primary agent in non-A, non-B transfusion hepatitis is HCV. Because HCV is found in human blood, blood units are screened for HCV with multiantigen assays detecting antibodies and with NAATs (see [Chapter 11](#)).

Case check 10.1

Based on the patient's clinical presentation and preliminary laboratory tests in the Case in Point, the physician suspected HIV infection. Laboratories generally screen for anti-HIV antibodies with an EIA. However, all reactive (positive) results must be confirmed by an additional serologic test.

Human immunodeficiency virus

The Centers for Disease Control and Prevention recommends that laboratories conduct initial testing for HIV with an antigen/antibody combination immunoassay that detects HIV-1 and HIV-2 antibodies and HIV-1 p24 antigen. Commercially available methods are based on EIAs and chemiluminescent immunoassays. Additional testing is not required for nonreactive specimens. However, a nonreactive result could occur early in the infection before antibodies were produced. Because antibody development in HIV infection has a wide variation depending on the patient, the infective dose, and the phenotype of the virus, seroconversion occurs an average 45 days after infection. For this reason, it is necessary to assay for HIV-specific proteins (antigens), such as p24 (a core protein)

or gp41 (a glycoprotein on the viral envelope). Screening kits detecting both the antibody and antigen, referred to as *fourth-generation tests*, are some of the most sensitive screening tests.

Specimens with a reactive antigen/antibody combination immunoassay result or a repeatedly reactive result must be tested with a supplemental immunoassay that differentiates HIV-1 antibodies from HIV-2 antibodies for confirmation. Supplemental assay methods include Western blot, indirect immunofluorescent assay, qualitative HIV-1 RNA assay, and HIV-1/HIV-2 antibody differentiation assay. The Western blot and indirect immunofluorescent assays detect HIV-1 infections later than other assay methods and therefore might produce false-negative or indeterminate results. The HIV-1 Western blot has also demonstrated cross-reactivity with HIV-2. For these reasons, the Western blot as a confirmatory test for HIV infection is falling into disuse. Specimens reactive on the initial antigen/antibody combination immunoassay and nonreactive or indeterminate on the individual HIV-1/HIV-2 antibody immunoassays should be tested with an HIV-1 NAAT. Assays vary in their sensitivity, specificity, and diagnostic utility and should be individually evaluated before use in a clinical laboratory.

The fourth-generation serology tests for HIV detect anti-HIV antibody and p24, the most abundant protein of HIV virions. Combining antigen detection with antibody detection increases the sensitivity of diagnosing a patient with HIV infection. Detection of p24 antigen is generally performed by capture EIA using a monoclonal antibody. One problem with this assay occurs during seroconversion. As the patient begins making antibodies to p24 antigen, immune complexes can form in vivo, which prevent the antigen and antibody from reacting with in vitro assays; this can result in false-negative results for both antigen and antibody detection assays.

Another problem with p24 antigen detection has been low sensitivity. Modification of the procedure, such as boiling the serum before testing to release immune complexes, has diminished the problem. However, their sensitivity remains less than that of NAATs. Blood units for transfusion are typically screened for antibodies to HIV, and a NAAT is performed for detecting viral RNA.

Case check 10.2

In the Case in Point, the patient's screening test for anti-HIV antibodies was reactive. Before a diagnosis of HIV infection can be assumed, a second confirmatory test must be performed. Previously, the Western blot assay was the confirmatory test most often used. Currently, the indirect immunofluorescent assay, qualitative HIV-1 RNA assay, or HIV-1/HIV-2 antibody differentiation assay are recommended.

Serologic diagnosis of fungal infections

Several methods have been used by clinical laboratories to identify antibodies in immunocompetent patients in response to fungal disease. Methods more commonly used today include ELISA, CF, immunodiffusion, and LA assay. The primary fungal diseases diagnosed by these means are histoplasmosis, coccidioidomycosis, and

paracoccidioidomycosis. Histoplasmosis is found primarily in the Ohio Valley. Coccidioidomycosis, or valley fever, is primarily seen in the San Joaquin Valley of California, and paracoccidioidomycosis is endemic from Mexico to Argentina. One fungal titer is not enough to be diagnostic because the people who live in an endemic area may have positive serologic tests from past exposure. A fourfold increase in titer is evidence of current infection. A travel history is mandatory when fungal disease is suspected.

The immunodiffusion and CF tests have been widely used for detecting antibodies during histoplasmosis. About 80% of patients will have a positive immunodiffusion test, and up to 95% will be positive by CF. However, the CF test is less specific because of cross-reactivity with aspergillosis, blastomycosis, tuberculosis, and other infections. The newer ELISA test has a reported sensitivity of 100% with acute disease and 90% in chronic disease.

The immunodiffusion assay for diagnosing coccidioidomycosis detects IgG and IgM. An ELISA is available that detects both IgM and IgG. IgM antibody is detected around the third week after infection; then the titer declines quickly. IgG antibody is detected in about 50% of infected patients by about 1 month. The test has been shown to have increased sensitivity, but there is concern about the test's specificity. It has been suggested that the EIA be used as a screening test and that any positive result be confirmed by a different method. LA assays are available from several manufacturers; however, the false-positive rate is higher than with other methods. Immunodiffusion and CF assays remain the most reliable. These two assays are also the most popular in diagnosing paracoccidioidomycosis.

Direct antigen detection assays

Antigen detection assays can provide rapid diagnosis of infectious agents, especially for those that are difficult or impossible to culture. Table 10.4 lists some infectious diseases that can be diagnosed by detecting antigens in clinical specimens. NAATs, discussed in Chapter 11, offer accurate, rapid results and are starting to replace direct antigen detection assays. As with any test procedure, laboratories must evaluate the tests for their situations.

Streptococcal pharyngitis

One of the most widely used applications of direct antigen tests, popularized in the 1980s, is for the detection of group A streptococci in throat swab specimens for the diagnosis of streptococcal pharyngitis. The main advantage of rapid direct antigen testing for streptococcal pharyngitis over a standard throat culture is that results are available while the patient is in the physician's office, allowing antimicrobial therapy to be given immediately instead of waiting 24 to 48 hours for culture results. This testing is more convenient for the physician and patient. Although antimicrobial treatment is critical for prevention of poststreptococcal pharyngitis syndromes, such as rheumatic fever and glomerulonephritis, early antimicrobial administration may also shorten the illness. In addition, early treatment may reduce secondary infections to close contacts, allowing the patient to return to school or work sooner. The most important reason for diagnosing

Table 10.4 Representative infectious diseases and direct antigen detection methods commercially available to detect them

Type of infection	Test methods
Bacterial	
Group A streptococcal pharyngitis	EIA, ICT, LA, liposome-enhanced immunoassay, OIA
<i>Clostridioides difficile</i> colitis	EIA, ICT
Legionnaires' disease	FA, ICT
Fungal	
Cryptococcal meningitis	EIA, ICT, LA
Pneumocystis pneumonia	FA
Parasitic	
Cryptosporidiosis	EIA, FA, ICT
Giardiasis	EIA, FA, ICT
<i>Trichomonas</i> vaginitis	ICT, FA
Viral	
Rotavirus gastroenteritis	EIA, LA
Hepatitis B infection	CIA, EIA
Respiratory virus infection ^a	EIA, FA, ICT
Herpes simplex virus infection	EIA, FA
Cytomegalovirus infection	FA
HIV infection	EIA

^aIncludes influenza, parainfluenza, and respiratory syncytial viruses.

CIA, Chemiluminescence; EIA, enzyme immunoassay; FA, fluorescent antibody test; HIV, human immunodeficiency virus; ICT, immunochromatographic test; LA, latex agglutination test; OIA, optical immunoassay.

and treating group A streptococci pharyngitis is to prevent the nonsuppurative sequelae. However, delay in initiation of therapy for a few days while awaiting culture results does not increase the patient's risk of developing such complications. Antimicrobial therapy begun as late as 9 days after the start of streptococcal pharyngitis will still prevent rheumatic fever.

More than 20 manufacturers produce diagnostic test kits for direct group A streptococcal antigen detection. These kits are based on immunoassays such as EIA and OIA. Generally, the tests contain reagents to perform an initial extraction of the group A carbohydrate antigen from the cell wall of the organism. This extraction is most commonly achieved by exposing the sample to nitrous acid, but it can be accomplished by enzymatic digestion (pronase). When the solubilized antigen preparation has been pH adjusted in a buffer, the specimen is tested according to the specific procedure of the manufacturer. EIA tests have become popular, particularly those that use membrane-bound antibody to speed up the reaction and facilitate the washing procedure. Because of the colored product, they are generally easier to read than LA tests. One variation of the membrane-bound EIA for group A streptococci uses colored, dye-filled liposomes bound to antibody as the detection reagent. These rapid tests are not as sensitive as culture, which remains the reference method. Rapid tests have a sensitivity of 80% to 90% in pediatric populations and about 90% in adult populations. Negative rapid tests in children and adolescents should be confirmed by throat culture.

Respiratory viral infections

Many respiratory viruses can be detected directly in both upper respiratory and lower respiratory tract specimens. The use of flocked swabs to collect upper respiratory tract specimens increases the quality and sensitivity of these assays. Flocked swabs have short, stiff nylon fibers extending from the shaft and act like a minibrush. Rapid techniques for detecting RSV, influenza A and B viruses, parainfluenza viruses 1, 2, and 3, and adenovirus include the immunofluorescent assay, immunochromatographic assay, and EIA. These assays may be used for direct detection or to confirm viral isolation in conventional cell culture tubes or shell vials. There is, however, great variability in the sensitivity and specificity of the tests. NAATs are gradually replacing antigen detection methods.

Of particular importance is the direct detection of RSV by fluorescent antibody or EIA and OIA in nasopharyngeal samples. The EIA and OIA are preferred over the DFA test because they are more rapid and less technically demanding. DFA tests have a reported sensitivity of 84% to 100% and a specificity of 86% to 100%. Rapid direct detection is important because RSV causes serious lower respiratory tract disease (bronchiolitis and pneumonia) in young children, often requiring hospitalization. Rapid results can help in making cohorting decisions on hospital admission. Also, it is difficult to maintain viral viability during transport; therefore it can be difficult to recover RSV in culture.

Influenza is another respiratory tract infection in which a rapid diagnosis is important for treatment. Many direct antigen tests with various formats are available for detecting influenza A or influenza A and B viruses. Generally, these tests have very good sensitivities and specificities, although sensitivity is lower in adults. Several formats are used including EIA, DFA, and ICT. Many assays require 2 hours and have sensitivities ranging from 50% to 80%. Several assays are characterized as rapid, providing results in 30 minutes or less, and are Clinical Laboratory Improvement Amendments waived. Two Clinical Laboratory Improvement Amendments-waived point-of-care tests are the QuickVue Influenza test (Quidel, San Diego, CA) and the FLU OIA (ThermoBioStar, Waltham, MA). These tests can be performed on numerous clinical specimens, such as nasal, nasopharyngeal, and throat swabs. Their sensitivity and specificity differ depending on the clinical specimen.

Bacterial meningitis

For years, clinical laboratories have regularly used antigen detection testing of CSF and other body fluids to detect organisms causing bacterial meningitis. Techniques have included EIA, coagglutination, and LA assays. The bacterial agents detected in commercially available kits include *H. influenzae* type b, *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *S. agalactiae* (group B *Streptococcus*). The sensitivities of many of these assays are very low, so routine use is no longer recommended. An exception is the Binax NOW *Streptococcus pneumoniae* Antigen Card (Alere). A study found that the ICT was positive for 68 (99%) of the 69 culture-confirmed pneumococcal meningitis cases and negative for 124 (99%) of 125 culture-confirmed meningitis cases caused by other bacteria. Multiplex polymerase chain

reaction (PCR) is generally considered more sensitive than culture and antigen detection.

The organisms causing meningitis often produce a bacteremia before invading the central nervous system. The organism and antigens may be detected in the serum before or at the same time as they are found in the CSF. Circulating organisms and soluble antigens are trapped, degraded, and released by phagocytic cells of the liver and spleen. These products are cleared from the body by filtration in the kidney and excreted in the urine; thus urine can be examined for the presence of antigens in addition to CSF in suspected cases of meningitis. Urine is also useful as a test specimen either alone or in addition to serum in cases of bacteremia and focal infections other than meningitis, such as pneumonia caused by *Legionella pneumophila*. Urine can be concentrated by ultrafiltration to increase test sensitivity.

The clinical utility of antigen tests for diagnosing meningitis varies with several factors including the manufacturer of the kit, the specific organism, and the type of specimen tested. In many clinical laboratories, the use of these tests is reserved for very specific diagnostic testing requirements. In most situations, Gram stain is sufficiently sensitive to detect bacterial meningitis. Gram stain of direct smears or antigen detection tests must always be performed along with culture. Most importantly, appropriate antimicrobial treatment should never be withheld pending culture results in patients with negative antigen test results, in contrast with the setting of antigen testing for group A streptococcal pharyngitis. The greatest clinical utility of these antigen tests is probably testing specimens from patients who have received antimicrobial therapy before cultures were collected and in whom Gram stained direct smear was negative. In this situation, the likelihood of recovering the organism in culture is dramatically decreased.

Giardiasis and cryptosporidiosis

The classic ova and parasite (O & P) diagnostic method requires chemical extraction, concentration, and microscopic examination of stool specimens. Although often considered the reference method, microscopic examination is technically demanding, is time-consuming, and requires trained personnel. Studies indicate, however, that the sensitivity of microscopic examination compared with immunoassays is low. In settings not including areas of endemicity for intestinal parasites (e.g., the United States), studies have suggested that 95% of all clinically important parasites detected in the United States are either *G. duodenalis* or *Cryptosporidium* spp. Several DFA, EIA, and ICT kits are available for detecting antigens from these parasites as well as *Entamoeba histolytica*/*Entamoeba dispar* directly from stool. DFA assays are performed on concentrated specimens, whereas EIA and ICT do not require concentration. Generally, the assays can be performed on fresh stool or stool preserved in 5% to 10% formalin or other preservative. Some formats include one monoclonal antibody to detect one organism; other formats use separate monoclonal antibodies for both organisms. When testing by DFA assays (see Fig. 10.13), the difference in size between these two parasites allows the laboratory scientist to distinguish the two organisms visually when using both antibodies in one procedure. Many laboratories offer these tests to screen immunocompetent outpatients.

Although the sensitivity and specificity of the immunoassays vary by manufacturer, these kits generally have sensitivities and specificities greater than 90%. However, studies found sensitivities of 68% to 75% in some ICT assays for *Cryptosporidium*. In addition, a high rate of false-positive *Cryptosporidium* rapid tests prompted laboratories to report positive test results as probable instead of confirmed.

A problem with antigen detection assays is that only two or three organisms can be detected; other clinically significant intestinal parasites would be missed that might have been detected with microscopic examination. Testing algorithms usually are established so only patients with a history of travel or immunosuppression require a full O & P workup when the screening test is negative. DFA assays also can be used to check municipal water supplies for *G. duodenalis* cysts; however, PCR assays are more sensitive and are used more frequently instead.

Case check 10.3

Although HIV infection is generally diagnosed by antibody detection, EIA for the viral p24 antigen is used to detect active viral replication in blood. Its greatest value is in the diagnosis of early infections, before antibodies are detectable, and in neonates in whom serologic diagnosis is of little value because of maternal anti-HIV IgG from a seropositive mother.

Infections in immunocompromised patients

Antibody detection in immunocompromised patients can be problematic because of the poor immune response in these patients. Direct antigen detection tests are sometimes more valuable. *Cryptococcus neoformans*, CMV, and *P. jirovecii* are important pulmonary pathogens in patients with transplants, cancer, and AIDS. Cryptococcosis is a devastating systemic infection generally limited to immunocompromised patients. Infection occurs initially in the lungs and rapidly disseminates to the brain and meninges. Before the advent of AIDS, disseminated cryptococcal infection was rare. Traditionally, the diagnosis of cryptococcosis meningitis has been based on culture of CSF or the presence of encapsulated yeast cells in India ink preparations of CSF. Antigen testing of CSF, performed by LA, EIA, or ICT, is considerably more sensitive than India ink direct examination of CSF. Antigen detection methods use polyclonal or monoclonal antibodies to the capsular (polysaccharide) antigen and are easy to perform. Quantitative antigen detection, which consists of titrating CSF antigens by performing serial dilutions, is an important prognostic indicator of clinical response to antifungal therapy. Many of these assays can be used on CSF and serum.

The Cryptococcal Antigen Latex Agglutination System (CALAS; Meridian Diagnostics, Cincinnati, OH) uses rabbit polyclonal IgG. This assay is more sensitive than assays using monoclonal antibodies. RF can produce false-positive results; therefore the manufacturer recommends pretreatment with pronase to remove RF and other nonspecific interference. The Remel *Cryptococcus* Antigen Test (Thermo Fisher Scientific, Waltham, MA) uses mouse monoclonal IgM; this assay incorporates a protease treatment for serum to prevent false-positive results by RF. The Premier Cryptococcal Antigen Test (Meridian Diagnostics) is an EIA that uses polyclonal antibodies attached to microwells. It has the advantage

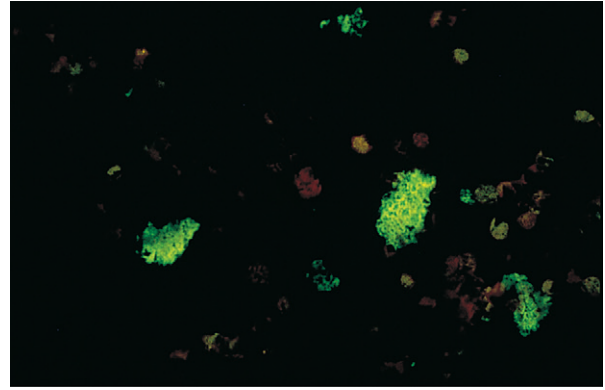


Fig. 10.28 Bronchoalveolar lavage showing *Pneumocystis jirovecii* using fluorescent antibody test.

of fewer false-positive results, does not react with RF, and can be run on many samples at once. An ICT method, the IMMY Cryptococcal Antigen Lateral Flow Assay (CrAg LFA), manufactured by Immuno-Mycologics (Norman, OK), can be used on serum, urine, or CSF. It uses monoclonal antibodies directed against capsular material detecting all four serotypes of *C. neoformans* complex including *Cryptococcus gatii*. A meta-analysis of published data found a sensitivity and specificity of greater than 95% for serum and CSF.

Early CMV structural proteins can be monitored by the CMV antigenemia assay. In this procedure, buffy coats from whole-blood specimens are stained by fluorescent antibody, and the number of positive polymorphonuclear cells per cells counted is reported. Increased numbers of fluorescently stained polymorphonuclear nuclei are indicative of increased risk of developing CMV pneumonia or other end-stage organ disease. Antigenemia tests are important to determine CMV infection in solid organ and hematopoietic cell transplant patients.

A fluorescent antibody procedure for *P. jirovecii* is approved for use on induced sputum and bronchoscopy specimens (Fig. 10.28). The most sensitive kits are those with monoclonal antibodies directed against antigens found in all forms of the fungus. In patients with high probability of pneumocystis pneumonia, a negative fluorescent antibody test on induced sputum should be followed by a fluorescent antibody test on a bronchoscopically obtained specimen. *P. jirovecii* culture is not widely available, making fluorescent antibody assays the only reliable method for its detection in the clinical microbiology laboratory.

POINTS TO REMEMBER

- Although the words *antigen* and *immunogen* are sometimes used interchangeably, an immunogen is a molecule stimulating an immune response, whereas an antigen is a molecule capable of binding to a specific antibody or T-cell receptor.
- Using antibodies to diagnose a recent infectious disease requires either demonstrating a fourfold increase in titer between acute and convalescent samples or distinguishing between IgG and IgM.
- Although monoclonal antibodies have the advantage of being more specific than polyclonal antibodies in detecting antigens, polyclonal antibodies are more sensitive.

- False-negative tests for antibodies to specific antigens can occur if the serum sample is collected too soon in the course of the infection, the patient is immunocompromised, or antibody concentration is extremely high (prozone phenomenon).
- False-positive tests for an antibody can be caused by many situations, including the presence of heterophile antibodies or RF.
- Precipitation reactions are based on the insolubility of immune complexes formed after binding of soluble antigen to soluble antibody.
- Agglutination assays use particulate substances, such as latex, to form visible clumps to detect immune complexes.
- Antigens or antibodies can be labeled with numerous markers, such as fluorochromes or enzymes, to detect antigen-antibody binding in vitro.
- In syphilis serology, screening tests use nontreponemal antigens, whereas confirmatory tests use treponemal antigens.
- Direct antigen detection methods offer accurate, rapid alternatives to culturing infectious agents.
- Except for cryptococcosis, direct antigen detection in CSF is insensitive and not recommended.

LEARNING ASSESSMENT QUESTIONS

1. Serum is collected from a patient taking high doses of biotin supplement. The primary health care provider requested thyroid function tests, which are determined using biotin-streptavidin noncompetitive assays. Which effect might the biotin supplements have on test results?
 - a. Falsely decrease antibody concentration
 - b. Falsely increase antibody concentration
 - c. Decrease specificity
 - d. No effect on antibody concentration
2. What is the significance of a high titer of IgM to cytomegalovirus (CMV) in a neonate?
 - a. Likely a false-positive test result
 - b. Indicates past infection in the mother
 - c. Indicates maternal infection during pregnancy
 - d. Indicates that the mother received a CMV vaccination during pregnancy
3. Which of the following is an advantage of using monoclonal antibodies instead of polyclonal antibodies in immunoassays?
 - a. Increased sensitivity
 - b. Increased specificity
 - c. Decreased avidity
 - d. Assays can be performed more quickly
4. Serial twofold dilutions of a patient's serum sample are prepared. The dilutions are tested for the presence of antistreptolysin-O antibody in a neutralization assay. Dilutions 1:1 to 1:16 show no hemolysis, whereas dilutions 1:32 to 1:128 exhibit hemolysis. How should the results be reported?
 - a. 1 Todd unit
 - b. 16 Todd units
 - c. 32 Todd units
 - d. >128 Todd units
5. In the indirect immunofluorescent assay for detecting an antibody, what is the conjugate?
 - a. Antibody-antigen complex
 - b. Fluorochrome-labeled antigen
 - c. Fluorochrome-labeled antihuman antibody
 - d. Fluorochrome-labeled antibody directed against the antigen
6. Which advantage does the Western blot assay have over other serologic tests?
 - a. Distinguishes IgG from IgM
 - b. Can be a waived point-of-care test
 - c. Uses fluorochromes instead of enzymes
 - d. Determines the presence of antibodies to several different antigens
7. A patient's serum sample was reported as reactive in a rapid plasma reagin (RPR) test. What should be done next?
 - a. Repeat the RPR test.
 - b. Perform another nontreponemal antigen test.
 - c. Perform a confirmative treponemal antigen test.
 - d. Report as reactive; no additional action is required.
8. Which of the following antibody titer results is suggestive of an acute infection?
 - a. Single serum sample: IgG 4, IgM 32
 - b. Single serum sample: IgG 16, IgM 4
 - c. Acute sample: total antibody 128, convalescent total antibody 32
 - d. Acute sample: total antibody 64, convalescent total antibody 64
9. The presence of antibodies that agglutinate sheep and horse red blood cells is suggestive of which disease?
 - a. Syphilis
 - b. Strep throat
 - c. Lyme disease
 - d. Infectious mononucleosis
10. When performing a latex agglutination assay to detect an antigen, why do some kit manufacturers recommend diluting specimens that are negative and then retesting?
 - a. Excess antibody to the antigen could be providing a false-negative result.
 - b. Excess antigen could be providing a false-negative result.
 - c. High concentration of complement could be providing a false-negative result.
 - d. High heterophile antibody concentration could be providing a false-negative result.
11. How does double immunodiffusion differ from single radial immunodiffusion?
12. List some causes of false-negative serologic test results.
13. How does passive agglutination differ from reverse passive agglutination?
14. Explain why current screening tests for HIV infection include anti-HIV-1 antibodies, anti-HIV-2 antibodies, and p24 detection.

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Applications of molecular diagnostics

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CHAPTER OUTLINE

Nucleic acid hybridization techniques, 228

Hybridization reaction variables, 229

Probe selection, 229

Hybridization formats, 230

Applications of nucleic acid hybridization techniques, 231

Nucleic acid amplification procedures, 231

Polymerase chain reaction, 232

Other nucleic acid amplification reactions, 238

Strain typing and identification, 241

Amplification-independent methods for strain typing, 241

Amplification-dependent methods for strain typing, 242

Molecular diagnostics testing in the clinical microbiology laboratory, 243

Sequencing, 244

Pyrosequencing, 244

Next-generation sequencing, 245

DNA microarrays and nanoarrays, 245

Proteomics, 246

Nanomedicine, 247

Bibliography, 249

OBJECTIVES

After reading and studying this chapter, you should be able to:

1. Describe the concept of nucleic acid hybridization and different parameters affecting the reactions.
2. Describe nucleic acid probes and how they are used for molecular diagnostic assays.
3. Explain the concept of nucleic acid amplification reactions and how these reactions are used in the clinical microbiology laboratory for molecular diagnostics.
4. Evaluate the use of nucleic acid amplification assays for molecular diagnostics.
5. Discuss the principle of the polymerase chain reaction (PCR) and the three types of PCRs.
6. Compare the methods of detecting PCR products.
7. Provide an overview of real-time PCR and the various real-time PCR detection methods.
8. Justify the uses of PCR in the clinical microbiology laboratory for molecular diagnostics.
9. Describe different amplification-independent procedures and how they are used in the clinical microbiology laboratory for molecular diagnostics.
10. Compare various strain-typing methodologies.
11. Discuss sequencing techniques, large-scale genomics, nanotechnology, and proteomics assays and their uses in the clinical microbiology laboratory for molecular diagnostics.

KEY TERMS

Agarose gel electrophoresis

Amplicon

Anneal

Annealing temperature (T_a)

Branched DNA (bDNA) assay

Denaturation

Dendrogram

Deoxynucleotide triphosphates (dNTPs)

DNA microarrays

DNA polymerase

Digital PCR

Droplet digital PCR

Dual-probe FRET

5' nuclease assay

Fluorescence resonance energy transfer (FRET)

Fluorophore

Housekeeping genes

Hybrid capture

In situ amplification (ISA)

In situ hybridization (ISH)

Invader technology

Melting curve analysis

Melting temperature (T_m)

Metagenomics

Molecular beacons

Multilocus enzyme electrophoresis (MLEE)

Multilocus sequence typing (MLST)

Multilocus variable-number of tandem repeat analysis (MLVA)

Multiplex PCR

Nanobiotechnology

Nested PCR

Northern blot

Nucleic acid hybridization

Nucleic acid sequence-based amplification (NASBA)

Oligonucleotide

Plasmid profile analysis

Plasmids	Ribonuclease H (RNase H)
Polymerase chain reaction (PCR)	Ribotyping
Primer annealing	Rolling circle amplification (RCA)
Primer extension	Sanger sequencing
Primer-dimers	Scorpion primers
Primers	Sequencing
Probe	Southern blot
Proteomics	Strand displacement amplification (SDA)
Pulsed-field gel electrophoresis (PFGE)	Stringency
Pyrosequencing	SYBR Green
Random amplified polymorphic DNA (RAPD)	Target
Real-time PCR	Template
Repetitive palindromic extragenic elements PCR (Rep-PCR)	Thermal cycler
Restriction enzyme	Transcript (messenger RNA)
Reverse transcription PCR (RT-PCR)	Transcription-mediated amplification (TMA)
	Uracil- <i>N</i> -glycosylase (UNG)

Case in point

A 26-year-old male patient noticed a small “pimple” on his right flank. Within a few days, the pimple developed into a boil (infection of a hair follicle). The area around the boil became inflamed; the skin was red, the boil began to swell, and the entire area was painful. Subsequently, he became nauseated, started vomiting, and had a low-grade fever. When he presented to the emergency department, he was ill enough to be admitted to the hospital.

The boil was drained, and the pus was sent to the microbiology laboratory. Gram-positive cocci in clusters were observed on a Gram stain of the purulent material. The following day, creamy white, β -hemolytic colonies were observed on sheep blood agar. The colonies were catalase-positive and positive by latex agglutination for coagulase. A preliminary report of “*Staphylococcus aureus* present, susceptibilities to follow” was entered into the hospital’s laboratory information system.

A real-time **polymerase chain reaction (PCR)** assay was run to determine whether the *S. aureus* isolate carried the *mecA* gene, the determinant of methicillin resistance in many staphylococci. In less than 2 hours, the real-time PCR assay determined that the patient’s isolate was positive for the *mecA* gene. An internal control for an *S. aureus*-specific gene was also used in the assay, confirming the identification of the isolate. The patient was treated with appropriate antimicrobial agents and recovered.

Issues to consider

After reading the patient’s case history, consider:

- The patient’s symptoms and presentation that may indicate the identity of the agent
- The use of rapid molecular diagnostic techniques that can aid in the identification of a causative agent and treatment of a disease
- The potential benefits to patient care and selection of a treatment plan that may result from using molecular diagnostic techniques
- The length of time that molecular diagnostic assays (on a scale of hours) took compared with conventional microbiological methods (on a scale of days)

Molecular diagnostics represents a significant advancement in the clinical microbiology laboratory over traditional cultures and is probably the fastest-growing sector in clinical laboratories. Molecular biology techniques aid in the diagnosis of infectious diseases and are widely used as testing and validating options by clinicians. This is primarily because of the high sensitivity and specificity of molecular diagnostic assays and their short turnaround time. It becomes particularly useful for agents that are difficult to culture or take a long time to grow on culture media. Molecular diagnostic assays provide clinicians with rapid answers for treatment options, thereby saving time in life-threatening infections. Many highly sensitive and specific, easy-to-use molecular assays have been approved by the U.S. Food and Drug Administration (FDA) and are on the market for use in diagnosing infectious diseases.

As the name suggests, molecular diagnostic assays detect the presence of molecules such as nucleic acids (i.e., deoxyribonucleic acid [DNA] and ribonucleic acid [RNA]) and proteins in clinical samples and culture isolates. The types of assays used in molecular diagnostics include amplification of nucleic acids, sequence-specific hybridizations, and other assays that can specifically identify the target molecule. This chapter discusses these molecular-based techniques and their applications in the modern clinical microbiology laboratory.

Nucleic acid hybridization techniques

Nucleic acid hybridization is a technique first described in 1961 by Marmur and Doty. Most molecular diagnostic testing procedures use the concept of nucleic acid hybridization. Conceptually, nucleic acid hybridization refers to the formation of hydrogen bonds between pairing nucleotides—adenine (A) with thymine/uracil (T/U) and guanine (G) with cytosine (C)—of single-stranded DNA and/or RNA molecules that are complementary to each other. Under the right conditions, this forms a stable, double-stranded nucleic acid molecule. The resulting double-stranded hybrids may be DNA:DNA, DNA:RNA, or RNA:RNA, as depicted in [Fig. 11.1](#). This hybridization process is also called *duplex formation*. This coupling of complementary single-stranded molecules is a key component for many of the tests discussed in this chapter, including blotting methods, the PCR assay, and other nucleic acid detection techniques.

GTTTATCGTATAAAG
A CAAATAGCATATTC

GTTTATCGTATAAAG
B CAAAUAGCAUAAUUC

GUUUUACGUAAUAAAG
C CAAAUAGCAUAAUUC

Fig. 11.1 Three different oligonucleotide hybrids. **A**, DNA:DNA hybrid. **B**, DNA:RNA hybrid. The *top* strand is DNA, and the *bottom* strand is RNA; note the *Us* instead of *Ts*. **C**, RNA:RNA hybrid.

The two single-stranded nucleic acid molecules used in hybridization techniques are referred to by different terms. One of the strands is known as the **target** or **template**. The target strand is the DNA or RNA sequence that will be identified by the molecular diagnostic method used. The target nucleic acid molecule typically is immobilized on a solid support medium or suspended in solution. The other strand involved in hybridization methods is called the **probe**. The probe is usually a single-stranded DNA or RNA **oligonucleotide** labeled with a reporter molecule that can be detected visually or by an instrument. In essence, the probe is used to detect the target nucleic acid molecule. In most currently used molecular-based methods, the probe is produced synthetically to detect a specific target nucleic acid sequence of a given pathogenic organism. Probes are used for the detection of microbial pathogens in many different types of samples. Apart from microbiological diagnosis, probes are also used for gene expression analysis, identification of gene rearrangements, detection of point mutations, and other alterations at the nucleic acid level. Probes are an integral part of commercially available molecular diagnostic kits.

Hybridization reaction variables

Several variables affect the outcome of a hybridization reaction. These variables include the temperature, nucleotide base composition of the probe, length of the probe, probe concentration, degree of complementarity between the target and the probe, ionic strength (salt concentration), and pH. When one or more of these variables is not optimized for a hybridization assay, the hybrids may not form efficiently.

Temperature

Temperature is a variable that is probably the most easily controlled during hybridization. The stability of a given hybrid can be calculated by determining its **melting temperature** (T_m). The T_m is the temperature at which 50% of hybrids have formed and 50% of the single-stranded nucleic acid molecules are still dissociated. Generally, the T_m is dependent on the nucleotide composition of the probe, particularly on the percentage of guanine (G) and cytosine (C) nucleotides in the probe. This is often referred to as the G + C composition, or G + C ratio, of the probe. The T_m is dependent on the G + C ratio because three hydrogen bonds form between G and C, instead of the two hydrogen bonds that form between adenine (A) and thymine (T); the G-C pair is more thermodynamically stable than the A-T pair. The following formula is used for calculation of T_m of a sequence:

$$T_m = 2 \times ([\text{number of (A + T)}]) + 4 \times ([\text{number of (G + C)}])$$

Length of probe

Another aspect that affects the T_m is the length of the probe; in general, the T_m is lower for a shorter probe. Hybridization reactions tend to occur more rapidly for shorter probes than for longer probes. However, if the probe is too short, it can hybridize nonspecifically to unintended targets. An optimal length of at least 18 to 20 bp is used for probes to obtain specific hybridization.

Probe concentration

The probe concentration also affects hybridization reactions. Higher probe concentrations typically lower the reaction time by saturating all available probe target sequences. However, excessive probe concentrations could also promote nonspecific binding of the probe to nontarget sequences. The use of 1 : 2 to 1 : 3 molar ratios of target and probe is often the starting concentration for an optimal hybridization reaction.

Degree of target and probe complementarity

The basis of hybridization assays relies on the specificity of nucleic acid binding reactions. Most assays utilize a probe that has an exact complementarity sequence to the target nucleic acid. Such probes should not bind to nucleic acids from different strains of microorganisms that possess slightly different target sequences through point mutations. This **stringency**, or strictness of binding, is modulated by altering the hybridization parameters. Under optimal conditions, exact matches between the probe and target will hybridize first, whereas mismatches form duplexes more slowly. Increasing the length of the probe and lowering the hybridization temperature allow mismatches to form duplexes more readily compared with shorter probes and higher annealing temperatures. High hybridization temperatures and short-length probes prohibit the mismatch formation and make the reactions more stringent.

Salt concentration

Optimal concentration of salts is needed to stabilize the duplexes in the reaction. The salt concentration (the ionic strength) can also affect the stringency of a given hybridization reaction. The rate of a hybridization reaction will increase as the salt concentration increases, up to a threshold; past a concentration of 1.2M NaCl, the rate of the reaction becomes constant.

pH

The pH affects the stability of double-stranded nucleic acid molecules in solution. An alkaline pH promotes dissociation of double-stranded molecules, whereas acidic pH solutions can depurinate probes and target nucleic acid molecules. Thus a neutral pH is preferable for most hybridization reactions. Most hybridization reactions are done in buffered solution at neutral pH to ensure that pH changes do not alter the desired outcome.

Probe selection

Selection of the proper probe for nucleic acid hybridization reactions is just as important as the hybridization reaction method itself. Essentially, the function of the probe is to form a duplex (hybrid) with every complementary sequence available in the hybridization reaction conditions being used. Probes may be DNA- or RNA-based, although most often DNA is preferred because of its high stability and low cost compared with RNA. Probes must be labeled so a detectable signal is obtained upon their binding to the target. Initially, nucleic acid probes were labeled with radioisotopes. The radiolabeled probe often had to be prepared by the user, and

success of a hybridization reaction depended on the efficient incorporation of the radionucleotide label into the probe. In addition, these probes posed a health risk during use and generated radioactive waste that required special disposable protocols. Radiolabeled probes have been replaced for the most part by nonisotopic labels, including chemiluminescent (light-emitting) molecules, enzymes, and fluorescent dyes. Generally, nonisotopic labels have resolution and sensitivity that approach, match, or even exceed those of radionucleotide labels, depending on the manufacturer and process used. Also, nonisotopic labels do not require special handling and specialized waste disposal.

A probe may be end-labeled or internally labeled. An end-labeled probe has the label attached to the 5' or 3' end of the nucleic acid sequence. A 5' end-labeled probe can be synthesized by transfer of the probe via a kinase reaction, whereas a 3' end-labeled probe can be synthesized by using a terminal transferase enzyme. An internally labeled probe has the label incorporated at multiple nucleotides along the length of the nucleic acid sequence. This is often achieved by using a nick translation reaction. In this reaction, a dsDNA sequence is treated with DNase I enzyme, which creates nicks in the sequence. DNA polymerase removes nucleotides from the nicked sites and then incorporates labeled nucleotides at the sites. Internally labeled probes can also be purchased from manufacturers.

Hybridization formats

Hybridization reactions can occur on a solid support mechanism, in situ, or in solution. Solid support hybridization reactions were invented first and are still used today mainly for research applications. The most current diagnostic assays utilize in-solution hybridization formats.

Solid support hybridization

In solid support hybridization, a technique often also called *blotting*, the target nucleic acid is transferred to

and immobilized on a membrane composed of nitrocellulose or nylon. The solid membrane is often pretreated to reduce nonspecific probe binding to the membrane itself. The labeled probe is then hybridized to the immobilized nucleic acid, and washing steps are used to remove the excess probe and clarify the signal. Detection is based on the type of probe used; colorimetric probes enable visual determination of positive and negative reactions or quantitative results with an instrument measuring color intensity, while chemiluminescent probes require a detection instrument (luminometer). The luminometer gives a reading in relative light units (RLUs). Two examples of solid support hybridization techniques are the Southern blot and northern blot.

Southern blot

The **Southern blot** is named after the scientist, Edwin Southern, who developed this technique in 1975. In this technique, chromosomal DNA is separated by **agarose gel electrophoresis** and then transferred to and immobilized on a nitrocellulose membrane. Usually, this method is used after chromosomal DNA has been digested with a **restriction enzyme**, creating double-strand cuts in the DNA at specific nucleotide sequences. The DNA is first digested because chromosomal DNA is too large to separate in an agarose gel. Once the fragmented DNA has been separated and immobilized on a solid membrane, the membrane is incubated with a labeled probe that is specific for the target DNA sequence. The labeled probe hybridizes to the specific target DNA sequence and is detected, as depicted in Fig. 11.2. Southern blotting can be used to identify microorganisms, detect mutations, and type strains for epidemiologic investigations and for other purposes. Southern blotting is somewhat labor-intensive, often takes more than 1 day to perform, and is now rarely used in clinical microbiology laboratories.

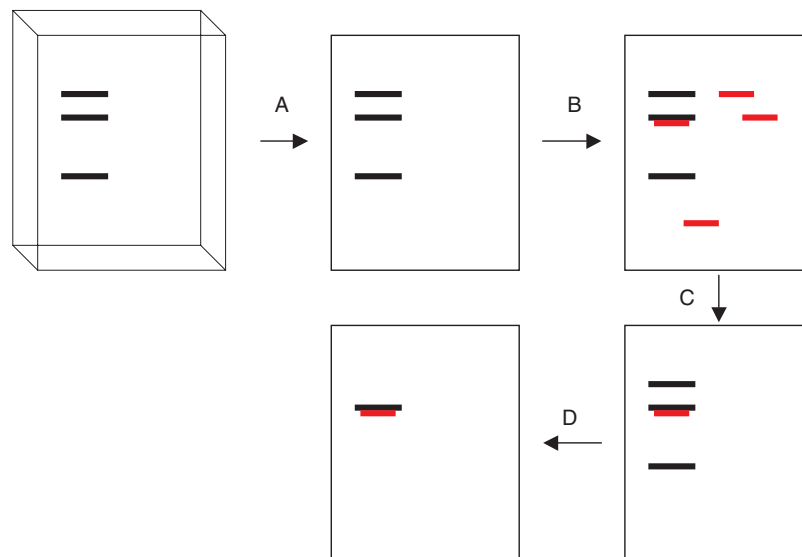


Fig. 11.2 Southern blot. **A**, Chromosomal DNA fragments separated in an agarose gel are transferred to a solid membrane. **B**, A labeled probe specific for a nucleic acid target is incubated with the separated DNA on the membrane. **C**, Excess probe is washed from the membrane, leaving the probe bound only to the appropriate target DNA. **D**, The probe–target DNA hybrid is detected.

Northern blot

The **northern blot** is used to detect RNA molecules employing the same principle as the Southern blot. The procedure to blot RNA onto a solid support mechanism was first described by Alwine and colleagues in 1977. This technique was named to be in sync with the previously established Southern blot (not named after a person). Northern blots can be used to determine the size of RNA molecules and semi-quantitate the amount of a particular RNA transcript. Just like Southern blotting, RNA molecules are separated in an agarose gel, transferred to a membrane, immobilized, and detected with a probe that hybridizes to the RNA species (nucleotide sequence) of interest. Detection is accomplished according to the type of label on the probe. One difference between Southern blotting and northern blotting is that a restriction enzyme is not used to digest the RNA before separation; RNA molecules are small enough to be separated efficiently by agarose gel electrophoresis. Like Southern blotting, northern blotting is also tedious and is rarely used in clinical microbiology laboratories.

In situ hybridization

In situ hybridization (ISH) was first described in 1969 by Pardue and Gall. In this hybridization method, DNA or RNA transcript can be detected directly inside cells and tissues with labeled probes, often fluorochromes or enzymes. The technique can be performed directly in tissue that has been embedded in paraffin. ISH can also detect nucleic acids in intact cells and chromosomal genetic material. In microbiology, ISH can be used to detect low levels of viruses in tissue specimens, such as human papillomavirus (HPV). Some laboratories will couple an amplification procedure such as PCR with ISH to increase sensitivity and specificity. This technique is called **in situ amplification** (ISA). Both ISH and ISA are highly sensitive and require technical expertise. These tests are not used routinely in clinical microbiology laboratories but are required when higher sensitivity is needed, such as in detection of trace amounts of viral genetic material in tissue specimens during early infection.

In-solution hybridization

In-solution hybridization, or liquid-phase hybridization, is the hybridization reaction most often used by clinical microbiology laboratories. Several manufacturers have developed useful assays that promote hybridization between a labeled probe and target nucleic acids in a liquid solution in tubes or in microtiter wells. Generally, detection methods for these commercial systems are chemiluminescence-based. Labeled probes are used during in-solution hybridization to confirm culture-based identification of bacteria and fungi and to rapidly identify pathogens.

Applications of nucleic acid hybridization techniques

Culture confirmation probe applications

The AccuProbe system by Hologic (Marlborough, MA) uses nucleic acid hybridization to confirm culture isolates

for several different organisms, including various mycobacteria, fungi, and bacteria. Regardless of the application, all AccuProbe tests use the same underlying principle. A single-stranded, chemiluminescent-labeled DNA probe is designed to hybridize to the target organism's ribosomal RNA (rRNA), forming a DNA:RNA duplex. A luminometer is used to detect these labeled duplexes. The RLU result for an unknown culture isolate is compared with a positive cutoff RLU value; any reading above the cutoff value is positive, and readings below are negative. The RLU cutoff value can change from one assay run to another. These assays can be performed on cultures obtained in solid or liquid media. The sensitivity and specificity for most of these assays approaches 100%, and most of these tests take about 1 hour to perform.

Probe-based assays that identify microorganisms directly from specimens

An example of a probe-based assay is the BD Affirm VPIII Test (BD Diagnostics, Sparks, MD) for patients with symptoms of vaginitis or bacterial vaginosis. The BD Affirm VPIII test detects *Candida* spp., *Gardnerella vaginalis*, and *Trichomonas vaginalis* in vaginal fluid specimens. This test uses a capture probe and a color development probe. Capture probes are attached to beads on a card; there is a separate bead for each organism.

Another example of a probe-based assay is the Accelerate PhenoTest BC Kit (Accelerate Diagnostics, Tucson, AZ). This test uses fluorescent in-situ hybridization (FISH) probes to directly identify six gram-positive bacteria, seven gram-negative bacteria, and two yeasts from positive blood cultures in about 90 minutes. This system combines antimicrobial susceptibility testing by bacterial growth to determine the minimal inhibitory concentration for several agents in about 7 hours.

Nucleic acid amplification procedures

Even though labeled probes can be used in nucleic acid hybridization assays to detect microorganisms in the clinical microbiology laboratory, they are not useful when the amount of target nucleic acid is low. There are three types of amplification assays: (1) target amplification in which the target molecule is amplified; (2) probe amplification in which the probe is amplified upon binding to the target; and (3) signal amplification in which the binding of the target and probe result in amplified signal generation.

Amplification procedures have many advantages over standard microbiological methods and over direct nucleic acid hybridization procedures. Many amplification assays combine rapid detection times with high sensitivity and specificity. In general, amplification assays are more sensitive than their nucleic acid hybridization counterparts because target nucleic acid sequences may not be present in high enough numbers to be detected by direct hybridization assays. In some cases, amplification tests can also be used to detect target nucleic acid in clinical specimens, and they present the advantage of speed over culture identification methods. Some amplification techniques also provide quantitative data and are useful to track the progress of a disease or provide the number of copies of microbial or viral nucleic acid present in a sample.

Disadvantages of amplification procedures include expense, the need for specialized testing and detection equipment, dedicated space to reduce contamination, and specially trained scientists. In addition, amplification procedures are highly targeted and specific, while culturing techniques allow growth of all organisms present in the sample. Sometimes, the high sensitivity of amplification methods leads to overdiagnosis as it can identify even trace amounts of residual nucleic acids of the pathogen after the infection has been eliminated. The most used amplification procedure by clinical laboratories is the PCR or some derivation of it. The PCR assay is based on target amplification. Other examples of amplification procedures include **nucleic acid sequence-based amplification** (NASBA), **transcription-mediated amplification** (TMA), **branched DNA (bDNA) assay**, and **hybrid capture**.

Polymerase chain reaction

The technique of synthesizing DNA was first described in 1971 by Kleppe and associates, although Mullis and others at Cetus Corporation in California developed PCR into its current application in the early 1980s. Mullis eventually received the Nobel Prize in Chemistry for PCR, and the advent of PCR initiated a revolution in molecular biology and several other fields, including microbiology. PCR is valuable because it is simple, sensitive, and powerful. PCR can amplify a single copy of target DNA into an exponential amount of nucleic acid product over the course of 25 to 40 reaction cycles.

As shown in Table 11.1, each cycle consists of three steps: (1) **denaturation** of target DNA; (2) **primer annealing** to the target sequence; and (3) **primer extension**. PCR requires several components, including an enzyme commonly called **DNA polymerase** (properly called *DNA-dependent DNA polymerase*), a buffer for the polymerase, **primers** (oligonucleotides that **anneal** specifically to target DNA and prime or start the synthesis of new DNA strands), magnesium ions (in the form of magnesium chloride, needed as a cofactor for DNA polymerase), equimolar concentration of four **deoxynucleotide triphosphates** (dNTPs—dA, dC, dG, and dT), and a source of template DNA (the target).

In the first step, the PCR reaction mixture is heated to separate the strands of target DNA. The reaction is then cooled so the primers anneal to the separated target DNA strands. After this, primer extension occurs, which synthesizes new strands of DNA. The cycle then begins again with heating to separate (melt) all of the strands of DNA (including the newly synthesized strands). The target DNA is exponentially amplified over multiple cycles (25 to 40) of these three reaction steps. Fig. 11.3 shows one cycle of PCR.

Table 11.1 Three steps in a polymerase chain reaction	
Step	Purpose
Denaturation	Separates double-stranded DNA
Primer annealing	Anneals primers to target DNA
Primer extension	Synthesizes new strands of DNA
DNA, Deoxyribonucleic acid.	

The original PCR method described was labor-intensive and used two separate heat blocks and the Klenow fragment from *Escherichia coli* as the DNA polymerase. Target DNA is denatured at temperatures higher than 90° C, so one heat block was set to 95° C. The other heat block was set to 30° C, the temperature at which the Klenow fragment from *E. coli* functioned best, to anneal the primers. The amplification reaction then proceeded by manual transfer of the reaction tube from heat block to heat block for several cycles; in addition, a new Klenow fragment had to be added after every cycle because it denatures at 95° C. In 1986, an instrument called the *thermal cycler* was developed; it is a heat block that cycles rapidly between temperatures so individual heat blocks are not required. In 1988 a heat-stable (thermostable) DNA polymerase from the thermophilic bacterium *Thermus aquaticus* was used in PCR. This was a major breakthrough in that the polymerase had to be included only at the beginning of the reaction and did not have to be added after every cycle. Several variations of standard PCR have been described, including **reverse transcription PCR** (RT-PCR), **multiplex PCR**, and **nested PCR**.

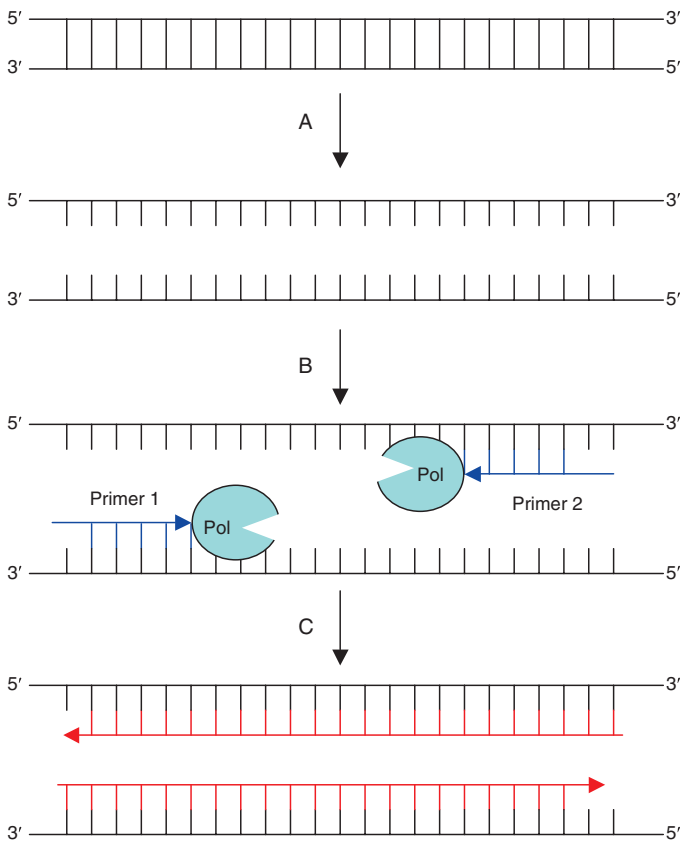


Fig. 11.3 One polymerase chain reaction cycle. **A**, Template DNA is denatured by heat to yield two single-stranded DNA strands. **B**, The temperature of the reaction is lowered, and the two single-stranded primers (primers 1 and 2, blue) anneal to the template DNA strands, DNA polymerase (blue spheres, Pol). **C**, The temperature of the assay is raised to 72° C, and DNA polymerase adds nucleotides to the primers to synthesize new double-stranded DNA molecules (new DNA, red) in the primer extension step. The new DNA is then used for the next cycle.

Case check 11.1

The Case in Point illustrates a common type of *Staphylococcus aureus* infection, a boil. Although rapid identification of infectious agents by the clinical microbiology laboratory is always important, it is even more important with *S. aureus* to determine whether or not a particular isolate is methicillin-resistant. Methicillin-resistant *S. aureus* isolates are more difficult to treat than methicillin-sensitive strains. One of the primary advantages of using a rapid molecular method such as PCR to determine methicillin resistance is the speed offered by PCR; an answer can be obtained with PCR in about 1 hour compared with about 24 hours for standard antimicrobial susceptibility tests.

Contamination prevention

Because PCR is so sensitive, small amounts of extracted nucleic acid or carryover amplicons from previous PCR assays can contaminate future PCR assays. This can result in false-positive results. Many laboratories use biological safety cabinets or dead-air boxes to set up molecular assays away from other clinical specimens. To prevent contamination, all equipment and work surfaces must be thoroughly cleaned with, at a minimum, 10% bleach or 70% alcohol or both before processing and after setting up an assay. Many laboratories clean equipment and work surfaces with products such as DNA Away (Thermo Fisher Scientific, Waltham, MA) to prevent carryover contamination from previous reaction products.

In addition, aerosol-resistant pipette tips should always be used for all amplification procedures. It is important to always wear gloves and to frequently change them. Dedicated laboratory coats should be used in each work area as well. All sample and reagent tubes should be capped when they are not being handled. Some laboratories take routine samples from workspaces and equipment to determine contamination (wipe tests). Such contamination screenings should be adopted into the laboratory's quality assurance program. The samples are used as a template in PCR assays; screening can occur at a rate determined by the laboratory (e.g., on a quarterly basis).

Polymerase chain reaction product analysis

Analysis of PCR amplicons can be accomplished by different procedures. In the past, analysis by agarose gel electrophoresis with ethidium bromide staining was popular. Agarose gel electrophoresis takes 1 to 2 hours to separate PCR products, generates hazardous waste (e.g., ethidium bromide), and requires electrophoresis apparatus (Fig. 11.4) and an imaging system. Although agarose gel electrophoresis is still important in research laboratories, nearly all PCR assays used in clinical microbiology laboratories use **real-time PCR** to analyze PCR products.

Real-time polymerase chain reaction

Real-time PCR was a major breakthrough for the detection of PCR products. The method was developed in the early 1990s by Higuchi and coworkers. Amplicons are detected as they accumulate in real time after each PCR cycle. This is accomplished by increase in a readable fluorescent signal

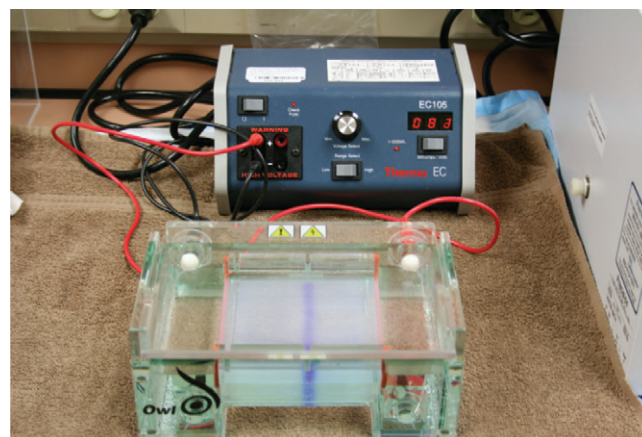


Fig. 11.4 Agarose gel electrophoresis. An agarose gel is shown submerged in running buffer in a gel box; next to the gel box is a voltage source that supplies the electric current to separate nucleic acids. The blue loading dye is visible in individual samples and permits visualization of the samples as they migrate through the agarose gel matrix.

as the reaction proceeds. Therefore the PCR can be monitored as it happens as opposed to standard PCR using an agarose gel in which amplicons are detected at the end of the entire procedure. Real-time PCR does not require an agarose gel, it usually does not accumulate hazardous waste, and the imaging system is part of the real-time instrumentation. Another benefit to real-time PCR is that the reactions occur in closed tubes that do not have to be opened for detection. Thus there is a much smaller chance that an amplicon from a real-time PCR assay will contaminate equipment, reagents, and workspaces.

Another advantage is that real-time PCR is extremely rapid compared with standard PCR. Many real-time PCR assays yield positive results in 30 to 40 minutes compared with about 4 to 5 hours for standard PCR. For example, denaturation time for real-time PCR is usually a few seconds compared with 15 to 30 seconds for standard PCR. Also, many real-time PCR procedures use primers with a T_m of approximately 72° C, so annealing and primer extension occur at the same temperature. This saves considerable time. Real-time PCR can be used to quantitate nucleic acids, which is useful for monitoring the progress of certain infections, such as those caused by human immunodeficiency virus (HIV), hepatitis B virus, and hepatitis C virus. Nearly all commercially available PCR assays use real-time PCR for detection.

Real-time PCR uses a fluorescent reporter dye, often in the form of labeled probes or beacons, sometimes called a **fluorophore**; a thermal cycler that uses an ultraviolet light source to excite the reporter, and a camera controlled by a computer system. Real-time PCR instruments can measure increases in reporter fluorescence as PCR amplicons accumulate, leading to rapid, accurate results. Fluorescent peaks are recorded by the computer system as fluorescence intensity versus PCR cycle number. Fig. 11.5 shows a real-time PCR fluorescence curve.

Fluorescence is measured by directly monitoring an increase in fluorescence or indirectly by a process called **fluorescence resonance energy transfer** (FRET). FRET is the transfer of energy from a donor dye molecule to an acceptor dye molecule. FRET also occurs between a fluorescent dye and a quenching molecule that keeps emitted light low until

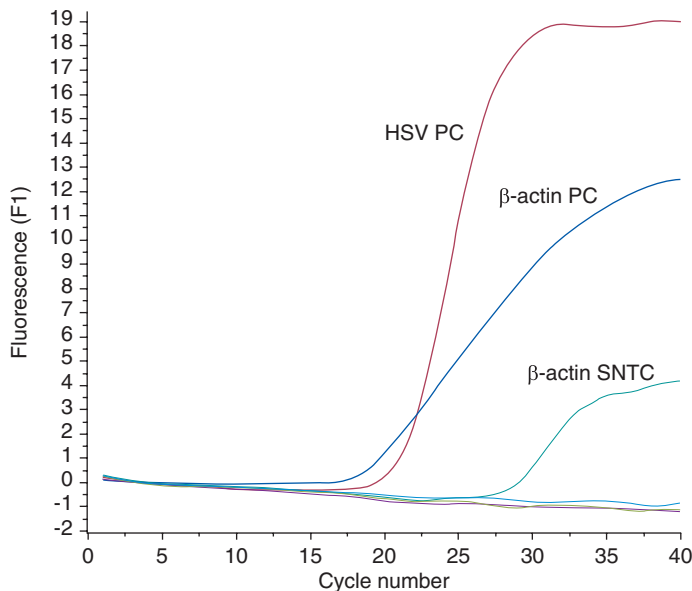


Fig. 11.5 Real-time polymerase chain reaction (PCR) data analysis for herpes simplex virus (HSV) from a human specimen. Fluorescent peaks are depicted as fluorescence intensity (*y*-axis) versus PCR cycle number (*x*-axis). An HSV-positive control (HSV PC) was assayed along with an internal control, β -actin positive control (β -actin PC), which should be present in all human samples. The HSV PC and β -actin PC have fluorescent peaks as does the β -actin assay from the unknown specimen, sample no template control (β -actin SNTC). The unknown specimen did not have a peak for HSV. In addition, a β -actin SNTC was assayed for both HSV and β -actin, and there were no fluorescent peaks for either one.

the fluorescent dye is released from the quencher. For FRET to occur, the two molecules must be in close proximity, within about one to five nucleotides of each other.

Some real-time PCR platforms can provide a **melting curve analysis** to determine assay results or verify the purity of the PCR product and determine whether primer-dimers are present. All dsDNA molecules have a T_m . This is determined by the length and GC content of the dsDNA hybrid. When a fluorescent dye is bound to the PCR product or attached to a probe bound to the PCR product, a fluorescent signal is detected by the real-time PCR platform. However, when the temperature is increased and reaches the T_m , the amount of fluorescence decreases as the probes and/or fluorescent dye dissociates from the PCR product. This decrease in fluorescence can be measured as a peak.

Real-time PCR platforms use different detection modalities, including the **5' nuclease assay** (sometimes called the *TaqMan assay*), **dual-probe FRET**, the **molecular beacons** probe, **Scorpion primers**, and intercalating dyes such as SYBR Green for detection of accumulated products. There are many commercially available real-time PCR platforms. A few examples include the 3M Integrated Cyclor (Focus Diagnostics, Fig. 11.6; now marketed by DiaSorin Saluggia, Italy, as the LIAISON MDX), the GeneXpert System (Cepheid, Sunnyvale, CA), the LightCycler 480 System (Roche Diagnostics, Indianapolis, IN), and the GeneAmp 9700 (Applied Biosystems, Pleasanton, CA). These platforms use rapid air exchange or rapid thermal conductivity around the reaction vessel to obtain rapid thermocycling; they also have a high surface-to-volume ratio of the PCR reagent mixture.



Fig. 11.6 3M Integrated Cyclor (Focus Diagnostics), another real-time polymerase chain reaction platform. (Image courtesy Steve D. Mahler.)

Case check 11.2

PCR can rapidly amplify the target sequence, but a decision must be made about which method will be used to determine how the amplicons generated are detected. Agarose gel electrophoresis allows the laboratory scientist to see stained amplicons, but this method adds another hour or more to the PCR process. Thus it is usually advantageous to use real-time PCR so amplicon-generated curves are displayed in real time. Results are generated more quickly with this method and can enable patients to be treated sooner.

5' nuclease assay (TaqMan)

The 5' nuclease assay uses the 5' exonuclease activity of some *Taq* polymerases (e.g., Gold DNA polymerase, Applied Biosystems) and TaqMan probes (Thermo Fisher Scientific). TaqMan probes are oligonucleotides about 18 to 22 bases long that are usually labeled on the 5' end with a fluorescent reporter dye and on the 3' end with a dye quencher. Fig. 11.7 illustrates the principle of a 5' nuclease assay. Fluorescence from the reporter dye is kept to a low background level because of the close proximity of the quencher by FRET (because of the conformation of the probe) in which the fluorophore donates energy to the quencher. The TaqMan probe for a specific PCR assay is designed to be complementary to a part of the internal region of the PCR amplicon during the primer-annealing step. During primer extension, the *Taq* DNA polymerase extends a complementary DNA strand from the primers using the template strand to which the TaqMan probe is annealed. The reporter dye is cleaved off by the 5' nuclease activity of the polymerase first, and then the entire probe is cleaved. Fluorescence increases as the fluorophore is removed from the immediate vicinity of the quencher. Fluorescence increases on a linear scale as more PCR product is synthesized during subsequent PCR cycles.

Advantages of the 5' nuclease method include (1) only specific PCR products are detected, (2) standard PCR protocols can be used, and (3) the hybridization and cleavage reactions do not interfere with production of the PCR product.

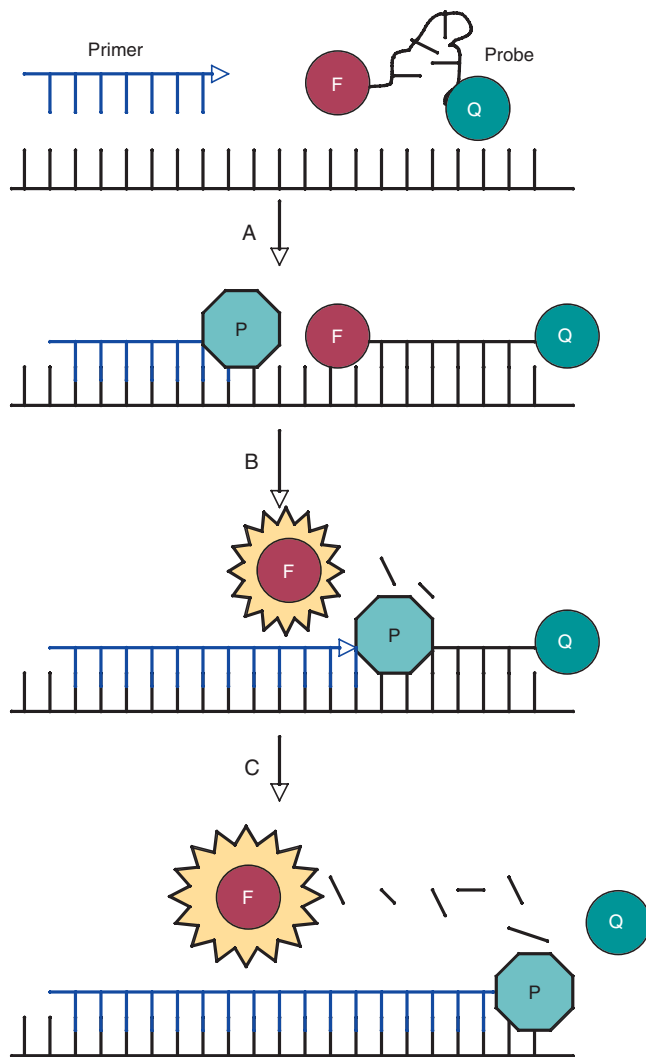


Fig. 11.7 5' nuclease assay (TaqMan). **A**, The primer and probe are annealed to the template DNA. DNA polymerase (P) starts to extend from the primer. **B**, DNA polymerase cleaves the fluorescent dye (F) from the probe and from the quencher (Q). Fluorescence is observed. **C**, As DNA polymerase continues to extend and synthesize a new strand of DNA, the probe is fragmented, and the fluorescent dye and quencher are fully released from each other. Fluorescence accumulates as fluorescent dye molecules are released.

One disadvantage is that specific probes must be designed and/or purchased for specific targets, and fluorescent-labeled probes are expensive compared with unlabeled oligonucleotides. Another disadvantage is that melting curve analysis cannot be performed when TaqMan probes are used because these probes are hydrolyzed during the amplification process.

Dual-probe fluorescence resonance energy transfer

This method is sometimes called the *dual-oligonucleotide FRET method* or simply the *FRET technique*. Dual-probe FRET uses two labeled probes, as shown in Fig. 11.8. One probe is labeled on the 3' end with a donor fluorescent dye, and the other probe is labeled at the 5' end with an acceptor fluorescent dye. The probes are designed to anneal head to tail to PCR amplicons. When this occurs, the two fluorescent dyes are brought into close proximity to each other, and FRET occurs. The light emitted by the real-time PCR platform excites the dye on the

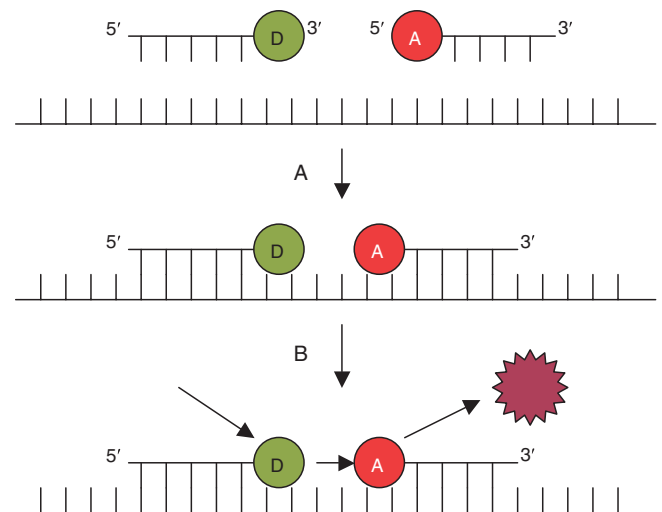


Fig. 11.8 Dual-probe fluorescence resonance energy transfer. **A**, Two labeled probes anneal to the polymerase chain reaction product as it accumulates. One probe is labeled with a donor fluorescent dye (D) on the 3' end; the other probe is labeled with an acceptor dye (A) on the 5' end. The two probes anneal to the PCR product head to tail. A single strand of PCR product is shown in this diagram after denaturation has occurred. **B**, The light source from the real-time PCR platform excites the donor fluorescent dye. The donor then transfers this energy to the acceptor dye. The acceptor dye is excited and emits fluorescent light, which is read by the instrument. Fluorescence increases as the PCR product accumulates.

first probe, and this dye then gives off fluorescent light at a longer wavelength. The energy given off by the 3' dye then excites the fluorescent dye on the second probe because the two dyes are so close together. This dye then emits fluorescent light at a longer wavelength than before, and this is measured by the instrument. The intensity of fluorescent emitted light is proportional to the amount of PCR product amplified during the reaction. Emission is detected only when the two probes anneal to their respective complementary sequences on the PCR amplicons. It is important to note that detection of fluorescence occurs only after the two probes have hybridized, so measurement occurs during the primer-annealing step. When the temperature is raised for the primer extension step, the two probes are displaced by *Taq* DNA polymerase, and FRET stops because the probes are no longer in close proximity.

The next measurement is made after the primer-annealing step in the next cycle. This is a specific technique that also allows melting curve analysis of resulting PCR amplicons. However, the technique is expensive and requires probe design skill.

Molecular beacons

Molecular beacons use short segments of DNA with a fluorescent-labeled reporter dye attached to the 5' end of the DNA segment and a quencher attached to the 3' end (Fig. 11.9). The middle section (loop) of molecular beacons has about 20 bases that are complementary to the amplicon. The two ends are designed with complementary DNA bases so they can base-pair with each other and form a hairpin structure with a loop; fluorescence from the reporter dye is quenched by FRET while the molecular beacon is in the hairpin structure because the dye and quencher are in close proximity. As PCR proceeds, the denaturation step separates template DNA, PCR product DNA (if present), and the

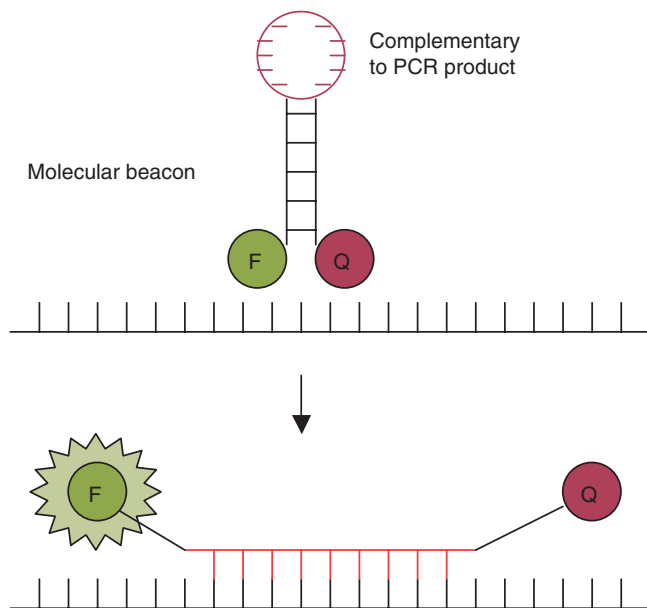


Fig. 11.9 Molecular beacons. The molecular beacon probe is a complementary hairpin loop structure. A fluorescent dye is bound to the 5' end of the hairpin, and a quencher (Q) is attached to the 3' end. A loop structure at the top of the molecule is complementary to the polymerase chain reaction (PCR) product formed. When the denaturation step of PCR occurs, the PCR product and molecular beacon probes dissociate; a single strand of a PCR amplicon is shown here. The beacon anneals to the PCR product formed, and then the fluorescent dye is removed from the quencher molecule. Fluorescence increases as the PCR product accumulates.

molecular beacon. When the temperature is lowered for the primer-annealing step, the molecular beacon complementary DNA anneals to a PCR amplicon, opening the hairpin and moving the quencher and reporter dyes away from each other; hence the fluorescence from the reporter dye is emitted. Molecular beacons that do not find the complementary target form hairpin loops and do not yield any fluorescence signal. Fluorescence is observed in a linear fashion as PCR amplicons accumulate. During the next denaturation step, the molecular beacons leave the target strand and are available to bind to the newly formed templates in the next round of annealing. Unlike Taq man probes, molecular beacons get recycled and reused and can also be used for melt curve analysis of the PCR products.

Scorpion primer

A Scorpion primer uses a single oligonucleotide to prime a specific sequence and detect accumulation of PCR product. [Fig. 11.10](#) illustrates Scorpion primers and their mechanism. Structurally, Scorpion primers resemble molecular beacons in that the nonhybridized form of the Scorpion primer is a hairpin loop. Scorpion primers have a fluorophore probe with a PCR blocker linked to the 5' end and a quencher attached to the 3' end of the hairpin loop structure so fluorescence is quenched when target is not present. In addition, a stem on the 3' end of the hairpin loop is complementary to a specific sequence of the target DNA, which acts as a primer for the DNA polymerase, so the Scorpion primer is linked to the synthesized product at the end of the first round of PCR. At the start of the second cycle, the hairpin structure of the Scorpion primer denatures, along with the template DNA. The hairpin probe sequence then hybridizes to the product that was just

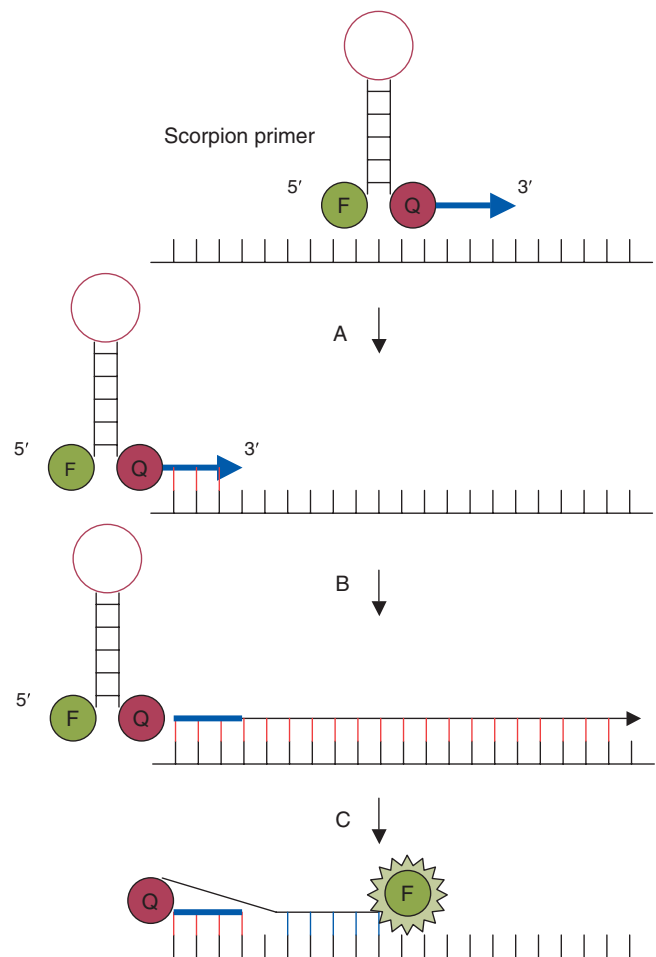


Fig. 11.10 Scorpion primer mechanism. **A**, A Scorpion primer is a probe and primer in one molecule. It is a hairpin molecule labeled on the 5' end with a fluorophore (F) and on the 3' end with a quencher (Q). A short priming sequence is also attached to the 3' end. The priming sequence anneals to the target DNA. **B**, DNA polymerase synthesizes a new strand of DNA from the short priming sequence. **C**, Denaturation occurs, and the newly formed DNA and Scorpion primer dissociate. An internal portion of the Scorpion primer is complementary to the product just formed. This portion anneals to the polymerase chain reaction product and separates the fluorophore from the quencher; fluorescence then accumulates.

synthesized. When this occurs, the fluorophore and quencher molecule become separated; accumulation of fluorescence indicates accumulation of PCR amplicons.

SYBR Green detection of real-time polymerase chain reaction products

SYBR Green is a fluorescent dye that binds to nucleic acids. SYBR Green I binds nonspecifically to dsDNA. The result of most real-time PCR applications is a dsDNA amplicon, so SYBR Green I is most often used in this application. Fluorescence increases as PCR product accumulates. SYBR Green I does not bind to denatured ssDNA strands at a high temperature. Some dye molecules begin binding to DNA during the primer-annealing phase, resulting in low-level background fluorescence. The dye then quickly binds to dsDNA during the primer extension step. As the cycles begin anew, fluorescence drops back to low background levels when the temperature is increased and denaturation occurs again. Thus detection by the real-time PCR platform occurs after every PCR cycle.

Using SYBR Green is less costly than using labeled probes, and it may be used for melting curve analysis. However, the dye binds to any nonspecific PCR products that form during assays and also binds to primer-dimers; thus it cannot distinguish among different PCR products. Note that melting curve analysis should be performed after SYBR Green detection is used to determine whether primer-dimers or nonspecific PCR products have formed. The melting curve temperature for primer-dimers and nonspecific products will be different than that for the amplified PCR products.

Other polymerase chain reaction procedures

Many different variants of PCR have been published in the literature or developed by various companies. Many of these PCR variations are used for specific purposes. Some of the more clinically used variant PCR procedures are discussed here, including reverse transcription PCR (RT-PCR), multiplex PCR, and nested PCR.

Reverse transcription polymerase chain reaction

RT-PCR uses an enzyme called *reverse transcriptase*, which is an RNA-dependent DNA polymerase, to synthesize a complementary strand of DNA (complementary DNA, or cDNA) from an RNA template. The resulting cDNA is then used as a template in a PCR assay using DNA polymerase (see earlier).

RT-PCR is often used in clinical microbiology laboratories to detect RNA viruses such as influenza virus and respiratory syncytial virus in clinical specimens. RT-PCR can be used to quantify the amount of virus in clinical specimens (e.g., HIV and hepatitis C virus). Other applications of RT-PCR include quantitative analysis of gene expression, detection of human genes involved in diseases, and detection of cancers in human specimens.

Multiplex polymerase chain reaction

Multiplex PCR is used to simultaneously detect two or more different targets in one sample in a single PCR reaction. In this technique, multiple primer sets for different targets are added together in the PCR reaction targeting different templates or different regions of the same template. Amplicon sizes from different genes should have a different length (base pair length) so distinct bands can be visualized by gel electrophoresis. Alternatively, distinct amplicons can be differentiated and visualized using primers coupled with different color fluorescent dyes.

Multiplex PCR can be broadly divided into (1) single-template PCR reactions that use a single template and several pairs of primers to amplify several regions within a template and (2) multiple template PCR reactions that use multiple templates and primers in the same reaction tubes. Most often, multiplex PCR is used to identify different species from a complex sample like contaminated water, food, or a clinical specimen. Several multiplex assays are available commercially to clinical laboratories for detection of multiple pathogens, mutant species, and viral variants.

Nested polymerase chain reaction

Nested PCR is a highly sensitive and specific PCR technique. Nested PCR consists of two different, consecutive PCR assays. The first reaction is a standard PCR assay using one set of primers. The amplicon produced from the first reaction

is then used as the target in a subsequent PCR assay. The second primer pair is complementary to an internal region of the amplicon derived from the first PCR assay. Amplicons are synthesized during this second PCR assay only if amplicons were produced from the first reaction, so this internal control mechanism serves as a marker of specificity.

Some clinical laboratories use nested PCR when the amount of starting template DNA is very low so the first reaction generates template DNA that is further amplified to a detectable amount. Also, some laboratories use a primer pair specific for a genus or viral family. If amplicons are present, as determined by agarose gel electrophoresis or real-time PCR, primers that determine species, strain, or type from a subsequent reaction are then used. For example, the first PCR assay could be used to ascertain whether a human herpesvirus is present in a sample. If present, primers specific for human herpesvirus type 1 and 2 could be used to determine the specific virus. One of the drawbacks to nested PCR is that it is not a closed system in that the first assay tube must be opened, so there is potential for contamination. Nested PCR is not used much in routine clinical microbiology laboratories.

Digital polymerase chain reaction

Digital PCR is a quantifiable, real-time PCR technique that translates exponential amplification data into digital signals. Digital PCR partitions a nucleic acid sample into many individual PCRs. In the newer digital PCR assays, called *droplet digital PCR assays*, the template and PCR reagents are dispersed into thousands of small droplets. Each droplet contains either only one starting target molecule or none, and a PCR reaction occurs in each droplet. Most of the droplets contain no template, and others contain individual target nucleic acid molecules. After multiple PCR amplification cycles, the samples are screened for fluorescence and recorded as 0 or 1. The fraction of fluorescing droplets is recorded. The absolute quantity of the nucleic acid can be determined by the count of positive signals (positive reactions). Reactions that have target nucleic acid are transformed into positive digital signals, and reactions with no target nucleic acid are transformed into negative digital signals. Digital PCR has been used in several applications, including genetic screening, oncology, and detecting low numbers of microorganism nucleic acid.

Applications of polymerase chain reaction in the clinical microbiology laboratory

With the commercial availability of real-time PCR platforms, many clinical laboratories use this method to diagnose bacterial, viral, fungal, or parasitic infections. Many assays have been described for antimicrobial drug resistance mechanisms as well. Several companies produce kit-based assays that are FDA approved for use in clinical laboratories. Some companies also produce kits called *analyte-specific reagents* (ASRs). ASRs are the active ingredients of an in-house test and are used by laboratories to establish and perform in-house (or so-called *home brew*) PCR tests. The manufacturer registers the product with the FDA, and laboratories use the ASRs in assays. Laboratories that use ASRs must establish and maintain performance of the PCR test and reagents. PCR assay patient results obtained with the use of ASRs can be reported

in a diagnostic setting. Laboratories that report results with ASRs must include a statement with the results, such as the following:

This assay and the test's performance characteristics were developed by [insert laboratory name]. This test has not been cleared or approved by the U.S. Food and Drug Administration (FDA). The FDA has determined that such clearance or approval is not necessary. This test is used for clinical purposes. It should not be regarded as investigational or for research. This laboratory is regulated under the Clinical Laboratory Improvement Amendments of 1988 (CLIA-88) as qualified to perform high-complexity clinical laboratory testing.

Several companies supply FDA-approved kits for their respective instrument systems for various organisms or antimicrobial-resistance genes. For example, Cepheid (Sunnyvale, CA) markets the GeneXpert system and offers PCR assays for various organisms including *Mycobacterium tuberculosis* that can be used directly on clinical specimens. Turnaround times are very rapid for these assays and can immensely improve patient care. Roche Diagnostics also offers various PCR assays on instrument systems. For example, the COBAS Amplicor is an automated PCR-based assay for *Chlamydia trachomatis* and *Neisseria gonorrhoeae*. The assay has FDA approval for testing endocervical swab specimens, urine from male patients, and urethral swab specimens from symptomatic male patients.

Other nucleic acid amplification reactions

Nucleic acid sequence–based amplification

NASBA is an isothermal procedure, which means that the reactions occur at a single temperature (usually 41° C), and therefore does not require a thermal cycler. NASBA is also referred to as *self-sustained sequence replications* (3SRs). The original method was described in 1989, but the method was not isothermal; instead, it used heat to denature the hybrids that formed during the procedure.

NASBA uses three enzymes: avian myeloblastosis virus (AMV) reverse transcriptase, **ribonuclease H** (RNase H), and T7 RNA polymerase. The amplification procedure results in multiple copies of RNA from the target sequence, as opposed to PCR, which results in multiple copies of DNA from the target sequence. The target nucleic acid can be DNA or RNA, although NASBA is most often used to detect RNA viruses such as HIV. During amplification, primers anneal to the target nucleic acid. One of the primers has a T7 RNA polymerase promoter added, and this primer initially anneals to the target sequence (Fig. 11.11). The reverse transcriptase generates a cDNA copy of the target, which results in a DNA:RNA hybrid. The enzyme RNase H then degrades the RNA, leaving the cDNA copy of the target; RNase H only degrades RNA from DNA:RNA hybrids. The second primer then anneals to the cDNA strand. Double-stranded DNA of

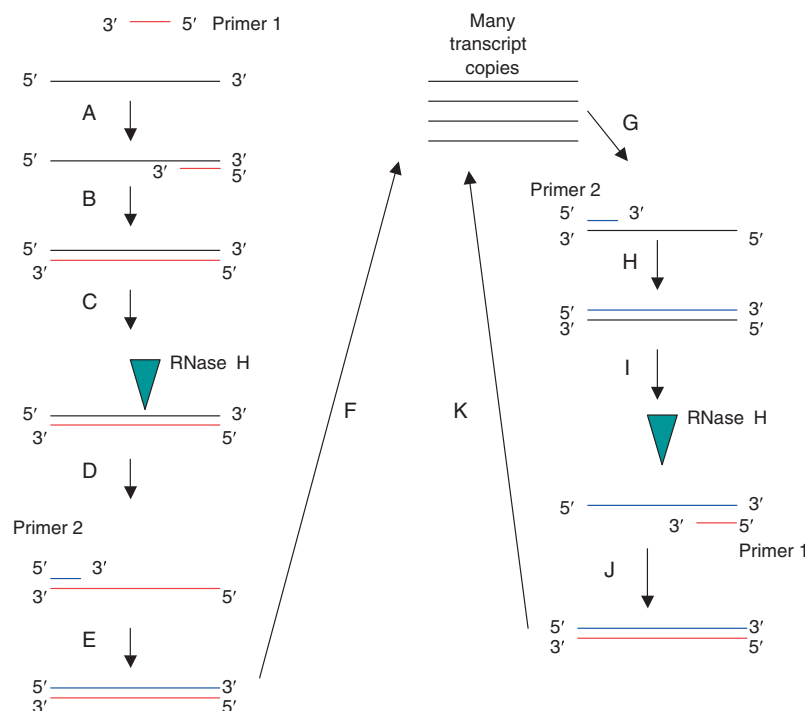


Fig. 11.11 Nucleic acid sequence–based amplification (NASBA). **A**, Primer 1 anneals to target RNA. **B**, Reverse transcriptase synthesizes a DNA copy of the RNA template. **C**, Ribonuclease H (RNase H) degrades the original RNA template. **D**, Primer 2 anneals to the DNA copy. **E**, Reverse transcriptase synthesizes another DNA strand, resulting in double-stranded DNA (dsDNA). **F**, T7 RNA polymerase synthesizes many copies of transcript using the dsDNA as a template. **G**, Primer 2 anneals to the synthesized transcripts. **H**, Reverse transcriptase makes a DNA copy of the transcripts. **I**, RNase H degrades the transcript copies, and primer 1 anneals to the DNA copies. **J**, Reverse transcriptase synthesizes new DNA strands, again resulting in dsDNA. **K**, The many copies of dsDNA are then used as a template by T7 RNA polymerase, which synthesizes even more transcript. This process continues in a loop.

the target is produced by the DNA-dependent DNA polymerase activity of AMV reverse transcriptase. The original primer with the T7 RNA polymerase promoter is integrated into this dsDNA copy of the original target nucleic acid. The T7 RNA polymerase then generates large amounts of transcript of the dsDNA. The transcript can be used again as a template for further amplification; instead, at this point, the second primer anneals to the generated transcript, and the DNA-dependent DNA polymerase activity of AMV reverse transcriptase once again makes a cDNA copy of these transcripts. The RNase H again degrades the RNA, and the first primer with the T7 RNA polymerase promoter anneals to the cDNA copies. The reverse transcriptase again synthesizes dsDNA, and the T7 RNA polymerase makes even more transcripts.

Thus amplification occurs continuously, and transcripts on the order of 10^9 are produced. The same method is used for DNA targets, although an initial denaturation step is required before NASBA can begin. NASBA is used to detect many types of viruses and microorganisms from clinical samples. The NucliSens EASYQ system (bioMérieux, Durham, NC) uses NASBA for the detection or quantification of viruses such as HIV-1 and HPV.

Transcription-mediated amplification

TMA, developed by Hologic, targets rRNA sequences of various microorganisms and produces large numbers of transcript using the same enzymatic processes as NASBA. The primary difference between the two methods is that TMA uses only two enzymes instead of three used in NASBA. In TMA, a reverse transcriptase with RNase H activity is used along with a T7 RNA polymerase to perform a reaction similar to the reaction in NASBA. The resulting amplified transcript is detected by Hologic's hybridization protection assay. Hologic Aptima assays utilize TMA on the Panther system. This automated system detects many different organisms, including *C. trachomatis*, *N. gonorrhoeae*, *T. vaginalis*, *Mycoplasma genitalium*, and herpes simplex viruses 1 and 2 from clinical specimens.

Strand displacement amplification

Strand displacement amplification (SDA) is an isothermal assay that can detect trace amounts of DNA or RNA. It can be used in multiplex reactions to detect several target sequences. The two phases of SDA are target generation and exponential target amplification. In the first phase, dsDNA is denatured and hybridized to two different primer pairs—the bumper and amplification primers. The bumper primers are shorter and anneal to the target DNA just upstream of the region to be amplified. The assay also utilizes a restriction enzyme and an exonuclease-deficient DNA polymerase. The primers bind followed by synthesis of dsDNA. The products then enter the second phase. The restriction enzyme nicks (makes a single-stranded cut) within the first primers. The polymerase then synthesizes a new strand, simultaneously displacing the original downstream strand, thus recreating the target sequence. The displaced strands can bind opposite strand primers, producing exponential amplification of the target sequences. Some of the single-stranded products bind to detector probes for real-time detection. The process continues amplifying the DNA. SDA has a reported sensitivity of as

few as 10 to 50 copies of target nucleic acid. It has been used to detect *M. tuberculosis*, *E. coli* O157:H7, and other infectious agents; hereditary diseases; and cancers.

Probe amplification reactions

These reactions amplify the labeled probe upon its binding to the target nucleic acid. Two types of probe amplification reactions include ligase chain reaction (LCR) and rolling circle amplification (RCA). The LCR is not used much today.

The RCA with padlock probes is an isothermal assay utilizing the ability of DNA polymerase to synthesize DNA on covalently closed circular templates to make a long linear strand containing multiple copies of a given sequence. RCA can replicate up to thousands of complementary amplicons into a single, long-stranded DNA. For probe amplification, the ligation of a padlock probe results in circularization of the target nucleic acid and thus triggering of an RCA reaction. Advantages of this method are resistance to many contaminants and excellent specificity. Disadvantages are the need for special instrumentation, and it is difficult to achieve quantitative detection. The padlock probe is used in diagnostic laboratories to detect several disease-related genes, Epstein-Barr virus (EBV) in human lymphoma specimens, influenza A H1N1 and H3N2 mutations, HIV, and more recently, novel coronavirus-2.

Signal amplification reactions

Several assays are currently used in clinical microbiology laboratories that exploit signal amplification for detection, rather than target or probe amplification. Signal amplification procedures used in clinical laboratories include bDNA detection, hybrid capture, and the Invader assay.

Branched DNA detection

Branched DNA (bDNA) detection is a sensitive signal amplification technique first described in 1991, and commercial assays are available for the quantification of RNA from hepatitis C virus and HIV and quantification of DNA from hepatitis B virus. This method uses oligonucleotide *capture probes* that hybridize target nucleic acid to a solid support mechanism, such as a microtiter tray (Fig. 11.12). Other probes, the *target probes*, anneal to a different region of the target nucleic acid than the capture probes. The target probes also hybridize to preamplifier probes. Amplifier probes then bind to the preamplifier probes, which forms a bDNA structure. Finally, probes labeled with alkaline phosphatase (AP) are added and hybridized to the complex; the complex is large, and many AP probes can hybridize to the bDNA structure.

When the AP substrate is added, chemiluminescence results, and light emission is detected by a luminometer and reported as RLUs. The amount of light signal is directly related to the amount of nucleic acid present in the sample. Standards are used with known concentrations of target nucleic acid, so a standard curve is established in light units during each bDNA assay. Unknown samples are compared with the standard curve to determine nucleic acid concentration. Although very strong signals are generated, there is an inherent risk of nonspecific results; therefore it is employed for quantitative viral loads in the clinical samples rather than for the detection of pathogens in the sample.

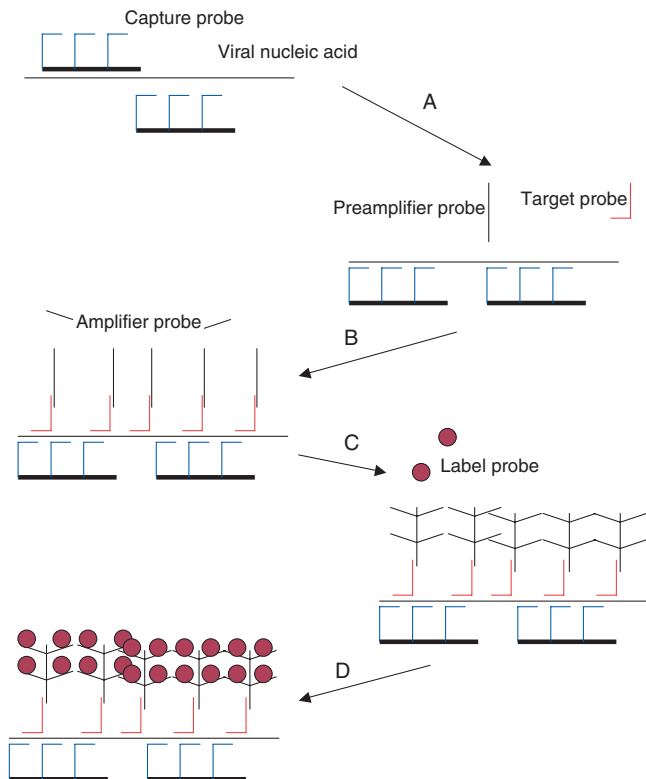


Fig. 11.12 Branched DNA (bDNA) detection. **A**, Capture probes attached to a surface anneal to target nucleic acid. **B**, Target probes anneal to nucleic acid and to preamplifier probes. **C**, Amplifier probes anneal to preamplifier probes, forming a bDNA structure. **D**, Label probes (with bound alkaline phosphatase [AP]) anneal to the bDNA structure. A large amplified signal is detected enzymatically when the AP substrate is added.

Hybrid capture

The hybrid capture method was developed by Digene in 1995 to detect HPV in clinical specimens. It is often done as in-solution hybridization. There are three steps: in the first step, the clinical samples are mixed with an alkaline denaturing solution that releases single-stranded viral nucleic acid into the solution. Then, an RNA probe specific for the target DNA is added to the sample, and a RNA:DNA hybrid forms (Fig. 11.13). A capture antibody is used to bind the hybrid onto the microtiter wells. Antibodies conjugated to AP are added to detect the captured hybrids. Multiple AP antibodies bind to each hybrid, resulting in signal amplification of approximately 3000-fold after a chemiluminescent substrate for AP is added. A luminometer reads light signals as RLUs. The initial assay, the Hybrid Capture I test, detected two HPV risk groups for cervical cancer: high-risk and low-risk HPV types. Testing was performed in tubes, and the method was a liquid hybridization assay. The Hybrid Capture II method (Qiagen, Frederick, MD) is automated and detects HPV types high risk for cervical cancer, *C. trachomatis*, and *N. gonorrhoeae*. A “detected” result for HPV does not identify which one of the high-risk 13 serotypes assayed for was detected. All three infectious agents can be detected from a single clinical sample in a microtiter plate format, and currently it is an FDA-approved assay for Pap smear specimens.

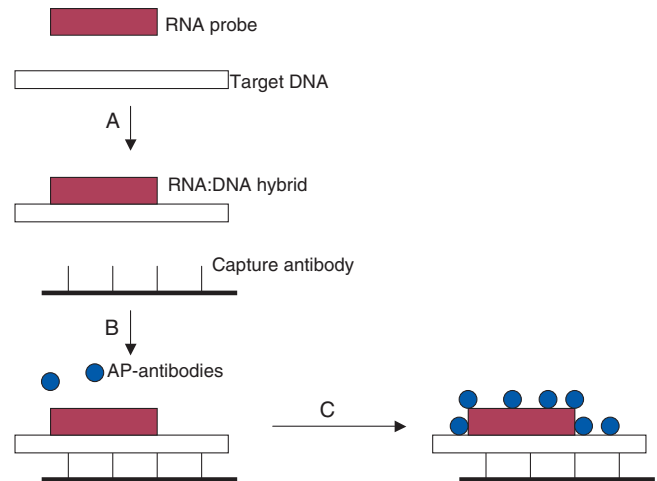


Fig. 11.13 Hybrid capture. **A**, RNA probes are annealed to target DNA, resulting in RNA:DNA hybrids. **B**, The hybrids are bound to capture antibodies attached to a solid support mechanism. **C**, Several alkaline phosphatase (AP)-antibodies bind to hybrids. The substrate is added for the AP and resulting light emission is captured by a luminometer.

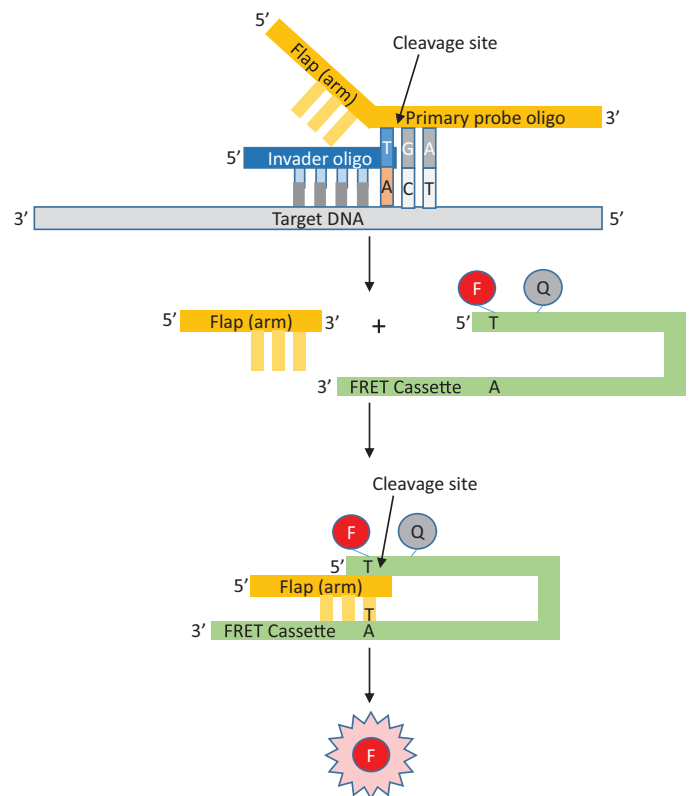


Fig. 11.14 Invader technology. A primary oligo and the Invader oligo anneal to the target DNA sequence, creating an overlap. The enzyme cleavage acts on this overlap at the cleavage site, releasing the 5' flap from the primary oligo. This 5' flap anneals to a fluorescence resonance energy transfer (FRET) cassette. The cleavage enzyme then acts on this structure, releasing a probe that is then detected.

Invader assay

Invader technology (Hologic) uses two different oligonucleotides that anneal to target DNA (Fig. 11.14). One is the primary probe oligonucleotide, and the other is the invader oligonucleotide. The primary probe oligonucleotide has a

flap, or arm, on the 5' end. When these two oligonucleotides anneal to the target DNA, they overlap. This forms a structure that is recognized by the enzyme cleavase. The 5' flap is cleaved by this enzyme at the overlap position. This newly released 5' flap then anneals to a FRET oligonucleotide called the *FRET cassette*. This creates another invasive structure that cleavase will use as a substrate. The interaction of cleavase releases the fluorophore and yields a signal. The probes in the reaction mixture are present in molar excess, so many probe interactions and signal-release events occur if the correct target DNA sequence is present. Invader chemistry is used with the Cervista HPV assay (Hologic).

Strain typing and identification

With the rise in the number of antimicrobial-resistant microorganisms, increases in the rates of toxin-producing bacteria, and spread of pathogenic microbes worldwide, there is a need for accurate, rapid, epidemiologic surveillance of these organisms. Standard techniques in clinical microbiology laboratories do not often provide the necessary resolution to distinguish types and strains. Instead, more refined techniques that separate related organisms from each other at the genetic level are necessary for epidemiologic investigations. Many typing methods have been described to determine genetic relatedness among bacteria, fungi, and parasites, and some provide better resolution than others. These techniques, often called *DNA* or *genetic fingerprinting*, are based on mutations that accumulate in biological organisms over time.

Strain typing techniques are often used to compare strains to determine whether a local outbreak is caused by a single strain type or by multiple strains. A single strain that is responsible for a disease outbreak may have a point source that can be targeted by public health services. Strain typing methods are also used to compare local isolates with worldwide isolates, which can show long-term spread of a strain or strains (clonality).

Whether testing local isolates or isolates from many locales, the technique chosen should have high discriminatory power or the ability to finely resolve different strains. Genetic fingerprinting techniques involve both amplification-dependent and amplification-independent methods. Amplification-independent strain typing methods usually involve the analysis of restriction enzyme fragments of chromosomal DNA. As strains diverge genetically over time, restriction enzyme sites on the chromosomal DNA will change as point mutations accumulate. When chromosomal DNA from different strains is digested with one restriction enzyme and separated by agarose gel electrophoresis, fragments of different sizes will be observed. These fragments are referred to as *restriction fragment length polymorphisms* (RFLPs). Comparisons of these RFLP patterns lead to strain typing, and evolutionary ancestors can be derived from the RFLP patterns. Similar RFLP patterns imply genetic relatedness.

Other amplification-independent typing techniques include Southern blotting, **plasmid profile analysis**, **pulsed-field gel electrophoresis** (PFGE), and **multilocus enzyme electrophoresis** (MLEE). Amplification-dependent fingerprinting methods use PCR and its variants discussed earlier. Known primers or arbitrary primers are used, depending on the technique. Commonly used amplification-dependent

methods include arbitrarily primed PCR (AP-PCR), also called **random amplified polymorphic DNA** (RAPD), **repetitive palindromic extragenic elements PCR** (Rep-PCR), and **multilocus sequence typing** (MLST).

Amplification-independent methods for strain typing

Plasmid profile analysis

This technique was one of the first methods used to type bacterial strains. **Plasmids** are extrachromosomal, circular DNA molecules found in variable number in the cytoplasm of many bacteria. Many bacteria carry antimicrobial-resistance genes, virulence genes, and other targets of interest on plasmids. The theory behind this technique is that a given strain with a plasmid profile will be unique from other strains with other plasmids. When a plasmid profile of different isolates is the same, it is possible that these strains are from the same clone.

Plasmid DNA is extracted from bacteria and separated by agarose gel electrophoresis. Unfortunately, because plasmids are extrachromosomal genetic elements that do not code for essential products, they are readily lost. Some plasmids also contain transposons, or so-called *jumping genes*, that are readily transferred to other bacteria. In addition, plasmids can exist in different forms in bacterial cells. Plasmids that have been cut or nicked will have different sizes on agarose gels than uncut plasmids. All of these factors can readily change the plasmid profile of a given bacterial isolate; therefore this technique is not often used in clinical diagnostic laboratories because of a lack of reliable reproducibility.

Southern blotting

Southern blotting, as described earlier, is sometimes used in epidemiologic investigations to analyze RFLP patterns. Chromosomal DNA is digested with a restriction enzyme, and the resulting fragments are separated by agarose gel electrophoresis. These fragments are transferred from the agarose gel to a nylon or nitrocellulose membrane, and a labeled probe is used to identify a specific target. One of the most popular targets is the DNA gene that codes for rRNA. When rRNA RFLP patterns are detected by Southern blotting, the technique is called **ribotyping**. The genes that code for rRNA are conserved in different species of organisms and appear in conserved positions of a species chromosome. Therefore ribotyping displays excellent reproducibility and discriminatory power.

A fully automated ribotyping platform that uses Southern analysis methodology is the RiboPrinter System (Hygiena, Camarillo, CA). The instrument automates the entire process, including lysing cells and data processing. Unknown patterns are compared with known patterns to identify strains. Some reports have indicated that other methods, such as PFGE and RAPD, have greater discriminatory power than automated ribotyping. However, automated ribotyping is advantageous for high-volume laboratories that evaluate large numbers of isolates. Other targets for Southern blot analysis are insertion sequences and transposons, which often contain genes involved in virulence and antimicrobial resistance. However, insertion sequences are not present in all strains of bacteria, so some strains are nontypeable.

Restriction enzyme analysis of chromosomal DNA

This technique resembles Southern blotting. Chromosomal DNA is extracted from isolates of interest and digested with one or more restriction enzymes. The enzymes used for digestion should cut the chromosomal DNA in several places so small and large fragments are produced. The resulting fragments are separated by agarose gel electrophoresis and transferred to a membrane. A probe to identify a specific sequence is not used. Instead, the DNA fragments (RFLP pattern) are stained and compared. The discriminatory power of this method is not high, and many of the fragments overlap and are difficult to distinguish from each other.

Pulsed-field gel electrophoresis

Schwartz and Cantor developed PFGE in the early 1980s. The technique is extremely popular and is perhaps the most well-known epidemiologic investigation method. It is used for strain typing and identification. A restriction enzyme is used to digest chromosomal DNA at a small number of sites along the chromosome. This results in large DNA fragments that are difficult to resolve by standard agarose gel electrophoresis. During PFGE, these large fragments are separated in an agarose gel by an angled electric field that periodically changes orientation. These changes in electric field orientation are pulses that successfully force the large DNA fragments through the agarose gel and separate them by size. A low-percentage agarose gel is used for the large DNA fragments, and low-melt agarose enables large fragments to migrate more easily. Relatively simple RFLP patterns result from this process, so comparisons are usually easy.

PFGE is also very reproducible and can theoretically be used on any organism. Fig. 11.15 shows representative PFGE patterns from different *S. aureus* strains. Generally, special equipment that can provide electric pulses must be used specifically for PFGE. Most PFGE assays take several hours (up to overnight) to efficiently separate the large DNA fragments produced in the restriction enzyme reaction. Most PFGE systems incorporate a chilling module within the system to cool the electrophoresis buffer and circulate it to improve separation. Without circulation and a chilling unit, the buffer will become very warm when separation proceeds for several hours—warm enough to potentially degrade the low-melt agarose gels used in PFGE. The warm temperatures also could produce wavy and difficult-to-interpret RFLP patterns. Among the various PFGE systems available is the GenePath strain typing system (Bio-Rad, Hercules, CA).

Multilocus enzyme electrophoresis

Unlike the other typing techniques described in this section, MLEE analyzes gene expression polymorphism. Analysis of protein polymorphisms by electrophoresis has been studied since the 1970s. MLEE involves extraction of proteins from isolates of interest, followed by electrophoretic separation and selective staining of these proteins. The expression of the protein's genotype is reflected in the position of the stained band, according to the protein's mobility. Mobility is determined by the net charge and structure of the protein. Two bands of the same protein in different positions after separation suggest two different conformations of the protein, in other words two alleles of the same gene.

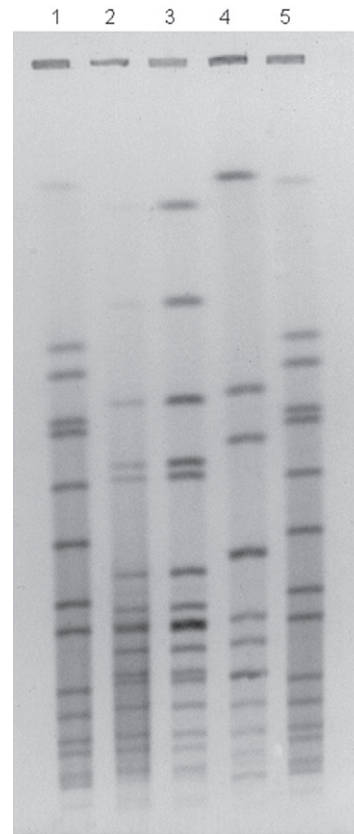


Fig. 11.15 Example of pulsed-field gel electrophoresis (PFGE) analysis of *Staphylococcus aureus* strains. Lanes 1 and 5, Known control strains of a *S. aureus* isolate. Lanes 2 to 4, Unknown strains from different patients with *S. aureus* isolates. Lanes 2 and 3 have the same banding pattern after PFGE and are the same strains. The lane 4 isolate is an unrelated strain.

MLEE is an excellent strain typing method. Unfortunately, the DNA sequence of the proteins separated during MLEE cannot be directly ascertained because different DNA sequences can result in the same protein because of the redundancy of the genetic code. In addition, two completely different proteins could have the same mobility and might be interpreted as the same proteins. Another problem is that interlaboratory comparisons are difficult. MLST, discussed later, was eventually developed because of these potential problems with MLEE (see later in this chapter).

Amplification-dependent methods for strain typing

Random amplified polymorphic DNA technique

The RAPD technique, also called *arbitrarily primed PCR*, was first described in 1990 by two different groups, Williams and colleagues and Welsh and McClelland. This is a popular method of DNA fingerprinting using small primers, approximately 10 nucleotides in length, with random sequences. These primers thus do not have a specific target. Instead, the random primers indiscriminately amplify chromosomal DNA during PCR cycles, which results in fragments of various lengths. After separation by agarose gel electrophoresis, different strains will have different fragmentation patterns.

The RAPD technique is a simple DNA fingerprinting method capable of providing high resolution. A single primer can be used in a RAPD PCR assay because the single random oligonucleotide can bind random targets on either strand of template DNA. However, the discriminatory power of just one primer is low. The discriminatory power increases if three or more primers are used, although this increases the assay time. Many laboratories use eight or more primers for RAPD DNA fingerprinting. One potential problem of the RAPD technique is interlaboratory agreement and reproducibility, although this method typically provides excellent intralaboratory reproducibility. RAPD analysis often yields some minor amplicons that exhibit low reproducibility in the same laboratory. Some researchers believe that PFGE has higher discriminatory power than RAPD analysis.

Repetitive palindromic extragenic elements polymerase chain reaction

The Rep-PCR technique is an amplification strain typing method first described in 1991 by Versalovic and associates. All organisms have repetitive DNA sequences—repetitive palindromic extragenic elements—that repeat throughout the genome. The unique DNA sequences that lie in between these palindromic repeats are amplified during Rep-PCR using primers specific for the repeat DNA. Rep-PCR results in fragments of various sizes, depending on the locations of the palindromic repeats. The length of DNA in between the repeats differs from strain to strain.

The discriminatory power of Rep-PCR is thought to be a little lower than for PFGE, although results seem to correlate well between the methods; some studies suggest that Rep-PCR has superior discriminatory power. The technique is easy to use and can be scaled up for several isolates, although RAPD assays are somewhat easier to perform. As always, a given laboratory should evaluate the methods to determine the best DNA fingerprinting procedure for the number of expected isolates and for the type of equipment and expertise available.

The DiversiLab System (bioMérieux) is an automated Rep-PCR instrument that uses proprietary Rep-PCR primers for different organisms. The system uses a microfluidic chip that evaluates Rep-PCR products. As many as 13 different isolates can be evaluated on one chip. Rep-PCR product fragments are analyzed with the Internet-based *DiversiLab* software to determine strain relatedness. Strain patterns can also be compared with the patterns obtained by other laboratories that use the *DiversiLab* system.

Multilocus variable-number of tandem repeat analysis

Like Rep-PCR, **multilocus variable-number of tandem repeat analysis** (MLVA) takes advantage of repetitive DNA sequences in genomes. MLVA amplifies regions of DNA that contain repeats. Repeated sequences in genomes tend to be unstable, so a given bacterial strain may have more repeats at one locus than a different bacterial strain of the same species. When there are different numbers of repeated sequences at a given locus, this is referred to as a *variable number of tandem repeats* (VNTRs). MLVA maps VNTRs among bacterial strains

by using PCR. This is a useful typing method because it produces quantitative data, it is easy to perform and results are easy to interpret, and it is reproducible.

Multilocus sequence typing

MLST is a derivation of MLEE, described in 1998 by Maiden and coworkers, that analyzes the sequences of genes. MLST is used to identify alleles by determining the internal sequences of **housekeeping genes**. Housekeeping genes code for proteins necessary for basic cellular functions and are constitutive genes (i.e., they are almost always expressed). Both eukaryotic and prokaryotic organisms have housekeeping genes. Bacterial housekeeping genes include the genes for 16S rRNA and dihydrofolate reductase. Because housekeeping genes are almost always expressed, they also make excellent controls for many molecular methods.

For a typical MLST assay, several loci are chosen that represent different internal regions of housekeeping genes. The PCR assay is used to amplify the DNA at each locus; primers are designed to act as a complement to highly conserved regions of the housekeeping genes. Once an amplicon is obtained, it is sequenced with an automated sequencer and given a unique number based on the sequence. Strains of a particular species can then be compared using the same loci. High resolution between strains is possible by comparing these DNA sequences or the numbers representing these sequences.

This method has achieved excellent interlaboratory comparisons; other laboratories merely need to use published primers for loci of the same species to compare data and determine the spread of strains. The method has achieved global recognition, and there are Internet-based databases of strains (<https://pubmlst.org/multilocus-sequence-typing>). The website provides protocols, information, and software for sequence analysis. A drawback of MLST is that it requires an automated sequencer, and the method is expensive compared with PFGE. However, of all methods described in this chapter for DNA fingerprinting of strains, MLST probably has the highest resolution and the greatest chance of interlaboratory agreement.

Molecular diagnostics testing in the clinical microbiology laboratory

Currently, numerous FDA-approved molecular assays are available for several organisms and antimicrobial-resistance genes. Several multiplex molecular assays can identify a large range of organisms by clinical syndrome or specimen site. For example, systems can detect several targets from positive blood cultures using multiplex molecular technology. The BioFire Blood Culture Identification 2 Panel (bioMérieux) can detect different bacteria, yeasts, and antimicrobial-resistance gene targets on one panel. The Verigene system (Luminex, Austin, TX) has both gram-positive and gram-negative blood culture tests for several bacterial and antimicrobial-resistance gene targets. The ePlex (GenMark Diagnostics, Carlsbad, CA) Blood Culture Identification Panels offer detection of many targets for gram-positive bacteria, gram-negative bacteria, and yeasts (including antimicrobial-resistance gene targets

for bacteria). The assays from all of these manufacturers can detect these targets in a relatively short amount of time (1 to 2 hours), depending on the system.

In addition to identification of organisms from positive blood cultures, multiplex assays are available for direct detection of organisms from respiratory, CSF, gastrointestinal, and other clinical samples. Indeed, there are many FDA-approved, highly sensitive and highly specific products on the market, and laboratories must evaluate which system and test is appropriate for their services and patient population.

One area that will continue to grow in clinical microbiology laboratories is nucleic acid sequencing. Despite advances in detection of organisms with large-panel multiplex assays, there are still difficult-to-identify organisms that may need to be identified by other means, such as DNA or RNA sequencing. As organism genome sequencing has increased, large-scale screening of nucleic acids and proteins became available. The information obtained from sequencing an organism's genome allows comprehension of cellular processes, protein associations, and interrelatedness of biological activities. Genomics research, coupled with the use of **nanobiotechnology**, has stimulated the invention of methods such as DNA microarray analysis and proteomics. Techniques such as **DNA microarrays** (and nanoarrays), **proteomics**, and **metagenomics** have the capability to revolutionize the analysis of microorganism populations and disease interpretation and treatment. Not all of these methods are ready for routine use in clinical microbiology laboratories; currently, many are primarily used as research tools.

Sequencing

DNA Sequencing is determining the order of nucleotides in a fragment of DNA; the chain termination method devised by Frederick Sanger is most frequently used. DNA sequencing has been available for years. Initially, the sequencing technique was somewhat labor-intensive, but it is now automated and has been applied to large-scale processes such as whole-genome sequencing. The human genome has been sequenced, as have the genomes of many other eukaryotic and prokaryotic organisms. Whole-genome sequencing is not a technique that clinical laboratories typically perform; however, clinical laboratories have started using small-scale sequencing techniques to identify microorganisms.

Many clinical microbiology laboratories now perform 16S rRNA gene sequencing to confirm the identity of problematic bacterial isolates. Other genes can also be sequenced to determine the identity of an isolate, such as *rpoB* (RNA polymerase B), which is often used to identify mycobacteria. To identify fungi by sequencing, a variety of targets have been used, usually within the rRNA gene complex, encompassing the 18S, 5S.8S, and 28S rRNA genes; the external transcribed spacer regions 1 and 2 that flank the rRNA genes; and the variable internal transcribed spacer regions 1 and 2.

In one popular method, a pair of primers is hybridized to template DNA prepared from bacterial cells, and PCR is performed to amplify a 500- to 1500-bp product. The library of PCR products is then purified with a commercial kit to remove excess primers and template DNA. Two reactions are performed during the next step, called *cycle sequencing*; one reaction uses a forward primer, and the other reaction uses a

reverse primer. In each reaction, the purified PCR amplicons from the previous PCR are used as the DNA template.

In the cycle sequencing reactions, the four standard deoxynucleotide bases (A, C, G, T) are included in the reaction mix, along with low concentrations of chain-terminating nucleotides of each dNTP. These chain-terminating nucleotides are also called *dideoxynucleotides triphosphates* (ddNTPs). Each ddNTP is labeled with a different fluorescent dye. When a labeled ddNTP is incorporated into the growing complementary strand of DNA, it terminates that strand; more dNTPs cannot be added beyond this point. A series of related DNA fragments are therefore synthesized, and these are terminated at the position at which the labeled ddNTP was incorporated. Fragments of every size are generated during this process. The generated fragments are purified to remove unincorporated chain terminators and are then separated by capillary electrophoresis in an automated analyzer. The sequence is thus generated based on fragment size, and a fluorimeter determines the dye on the end of each fragment. Each ddNTP has a different color fluorophore, enabling identification of the ddNTP on the end of each fragment.

Once the sequence has been determined, software is used to compare the generated sequence with a database of known sequences. A most likely identity can then be determined. When examining the relatedness of different species and strains of bacteria by sequencing, **dendrograms** are often used. Different methods of generating dendrograms include the neighbor-joining method, unweighted pair group method with arithmetic averages, and weighted pair group method with arithmetic averages. Generally, these different methods produce comparable results.

To determine the most likely identity of the unknown sequence, the investigator chooses similar organisms for comparison. For example, if the unknown isolate that was sequenced is an oxidase-positive, nonfermenting, gram-negative bacillus, several *Pseudomonas* spp. and related organisms may be chosen from the software database to compare them with the unknown isolate. In addition, an outgroup is chosen; this is a sequence against which all of the other similar sequences are compared. The outgroup should be somewhat related to the other organisms chosen, but still outside the group being studied.

Once the dendrogram has been generated, a horizontal line with a percentage is produced by the software at the top of the dendrogram next to the name of the method used to generate it. This horizontal line can be used as a rough measure of the degree of relatedness or difference between isolates in the dendrogram. To determine relatedness, the two horizontal lines of the two isolates in question are added and then compared with the top horizontal line percentage. Although this method is not completely accurate, it does provide a useful estimate of relatedness. See [Fig. 11.16](#) for a depiction of a dendrogram of different Enterobacterales species, generated with the *MicroSeq* software package (Life Technologies, Grand Island, NY). *P. aeruginosa* was chosen as the outgroup for this dendrogram.

Pyrosequencing

Pyrosequencing is a sequencing by synthesis technique that does not require labeled nucleotides or primers and does not require a postreaction electrophoresis step. It is a rapid

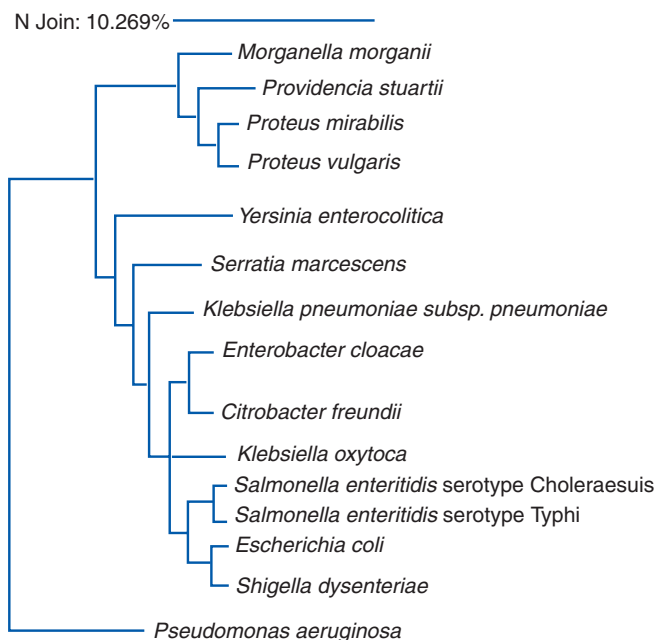


Fig. 11.16 Example of a dendrogram showing the genetic relationships among different Enterobacteriales species based on 16S ribosomal RNA gene sequencing.

sequencing technique that generates approximately 20– to 50–base-long sequences per primer, so this technique is best used for short sequences. Sanger sequencing is the best choice for longer DNA fragments.

Pyrosequencing uses the enzymes DNA polymerase, adenosine triphosphate (ATP) sulfurylase, luciferase, and apyrase and the substrates adenosine 5'-phosphosulfate (APS) and luciferin. A sequencing primer is hybridized to a single-stranded DNA template and incubated with the enzyme-substrate mix. The four different nucleotides are then added one at a time in a defined order. If the added nucleotide base-pairs with the DNA template, the DNA polymerase will incorporate the nucleotide with the release of pyrophosphate (PPi). In the presence of the substrate APS, ATP sulfurylase converts PPi to ATP. Luciferase then uses the newly formed ATP to convert luciferin to oxyluciferin, which releases light. The light is detected with a charge-coupled device camera. This is a quantitative and real-time process. Each light signal indicates that a nucleotide has been incorporated by DNA polymerase. The generated light is visualized as a peak, and the height of the peak indicates how many nucleotides were incorporated. The enzyme apyrase degrades excess nucleotides. After degradation, a new nucleotide is added, and the process begins again. The nucleotide sequence of interest is thus determined from the light peaks that occur during the nucleotide addition and incorporation process.

Pyrosequencing was used initially to determine sequences in mutation analyses of human DNA. However, the technique has also been used to identify microorganisms and to detect various antimicrobial-resistance genes in *M. tuberculosis*.

Next-generation sequencing

Next-generation sequencing (NGS) brings high throughput and accuracy to sequencing via automated platforms.

Several different platforms are available, such as the HiSeq and MiSeq systems from Illumina (San Diego, CA) and Ion Torrent (Thermo Fisher Scientific). Each of these technologies differs in the length of sequence that is read by the platform. In addition, the sequencing method, run time, and cost are different. Throughput (speed) is enhanced by NGS methods because numerous sequencing reactions can be run by the platforms in parallel, and the chemical reaction can be combined with signal detection.

DNA microarrays and nanoarrays

The term *DNA microarray* refers to a grouping at the micron level of DNA molecules attached to a solid support, such as silicon chips, glass, or plastic. A DNA microarray is sometimes referred to as a *DNA chip*, or *gene chip*. A DNA microarray gives investigators the potential to evaluate gene expression from an entire organism, or even from several organisms. DNA microarrays are often used to analyze transcriptional levels of genes during a particular disease. This technique has been used for many purposes, such as determining mutations, identifying new genes, monitoring response after treatment, determining binding sites for transcription factors, and identifying pathogens. For example, a DNA microarray could be used to simultaneously detect several pathogens. This could be applied to pathogens of interest in clinical and veterinary medicine, for public health issues, and for homeland security (e.g., screening for potential biothreat agents on one DNA chip).

A DNA microarray is constructed with special micro instruments that are capable of placing microscopic spots of DNA on a solid surface. Thousands or more can be placed on one chip. These spots of DNA are commonly termed *reporters*. DNA fragments from a specimen of interest are labeled with a fluorescent dye and incubated with the DNA on the chip. Unbound strands are removed by washing. A scanner reads fluorescence that develops only when a hybrid occurs. The hybrid that fluoresces is matched to the known map of the DNA chip. For example, an investigator may have a DNA microarray constructed with a single-stranded DNA sequence from many pathogenic respiratory bacteria.

Alternatively, RNA can be extracted from a patient sample, and reverse transcriptase is used to prepare large amounts of cDNA from each transcript. A fluorescent probe is enzymatically attached to the cDNA produced. The tagged cDNA is then incubated with the DNA on the chip and analyzed by a screener. A fluorescent signal from one DNA spot would indicate a hybrid and could imply a disease role for a particular organism. Amplification procedures can also be coupled to DNA microarrays for a potentially more powerful identification and analytic tool.

With the recent increase in the use of nanotechnology, nanoarrays (nanochips) have been developed. A nanoarray has molecules placed on a surface at defined locations, with nanometer spatial resolution. Like microarrays, nanoarrays are being developed for genomics and proteomics applications, such as organism identification. Nanotechnology applied to a chip does not need coupled reporters, as DNA microarrays do, and can screen more molecules; therefore nanoarrays are more sensitive and have higher throughput compared with DNA arrays.

Proteomics

Genomic sequencing has also led to an increased understanding of protein interactions and expression in cells. Proteomics is the study of proteins on a cellular level. Like genomics, proteomics is a large-scale process, but it is probably more complicated than analysis at the gene and transcriptional levels. Protein expression changes, for example, from cell to cell, among different disease states, during the life cycle of a cell, and during responses to changing environmental conditions. In addition, proteins with the same genetic origin can be vastly different as a result of alternative splicing of their coding RNAs or after posttranslational modifications.

The genome of an organism is the sum of the genetic material; the proteome of an organism is the sum of proteins found during all changing conditions for a cell. Thus the proteome is often larger and more complex than the genome. Proteomics is used to determine protein expression in disease conditions, such as cancers, genetic diseases, and other diseases, including microbial infections. For example, proteins identified in a certain disease state may become targets for new laboratory tests. In another example, therapy for a given condition may be based on the protein expression involved in the disease state.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

Matrix-assisted laser desorption/ionization–time-of-flight (MALDI-TOF) mass spectrometry is a technology that is used for rapidly identifying microorganisms, including fungi. This technique has been used for years in chemistry to analyze molecular molar masses. Microbial identification can be made in minutes from isolated colonies. Apart from a one-time, up-front instrument cost, MALDI-TOF analysis is very cost-efficient. Currently, two MALDI-TOF platforms are FDA approved for use in the United States: the VITEK MS (bioMérieux) and the MALDI Biotyper (Bruker, Billerica, MA; Fig. 11.17).

After an organism is recovered on solid media, a small amount of the colony is applied to a spot on a metal target (Fig. 11.18). An energy-absorbing chemical matrix solution is added to the colony material. The matrix solution consists of a crystallized chemical such as α -cyano-4-hydroxycinnamic acid, water, and an organic solvent such as acetonitrile. The organic solvent (and water) extracts proteins from the microorganisms on the target. The matrix solution is allowed to dry onto the colony material; the solvent will vaporize, and this results in the colony proteins being co-crystallized with the matrix chemical.

The metal target is then loaded into the ionization chamber of the MALDI-TOF mass spectrometer, and the co-crystallized organism protein/matrix lattice is pulsed with an ultraviolet laser. The activity of the laser causes the matrix to transfer protons to the colony material, producing singly protonated ions of the proteins, which gives these proteins a positive charge. The matrix also absorbs the laser light, converts it to heat energy, and is vaporized (desorption), along with the proteins, in nanoseconds. The ionized proteins then enter a flight tube. An electrode then accelerates the positively charged ions into a mass analyzer. The mass analyzer separates the ions by their mass-to-charge ratio. The amount of



Fig. 11.17 Bruker MALDI Biotyper system (Bruker). (Image courtesy Steve D. Mahlen.)

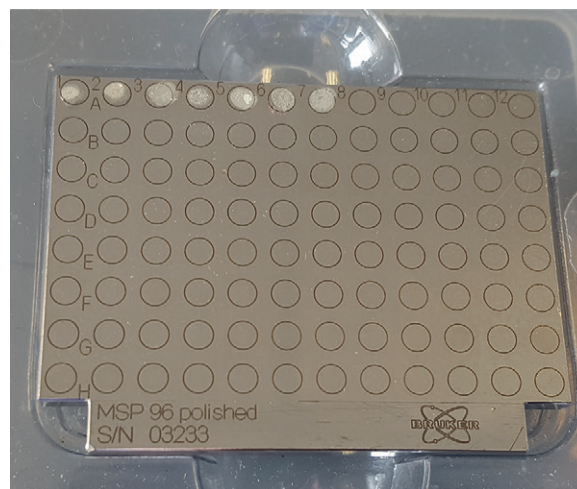


Fig. 11.18 Metal target, Bruker MALDI Biotyper system (Bruker). (Image courtesy Steve D. Mahlen.)

time it takes for the ions to move through the analyzer and be detected is the time of flight. Each protein generates a distinct signal, and every microorganism has a unique set of protein signals. A mass spectrum is thus generated for each microorganism and is unique for each species. Company-specific software is used to identify the organism based on a comparison with a reference database of spectra.

Metagenomics

It is estimated that only about 1% of all prokaryotes from most environments on our planet are culturable in the laboratory. Understanding the complexity and interactions of mixed microbial populations may yield valuable information that can affect human health and our association with the environment. For example, research has shown that the trillions of bacterial cells that make up the human gut microbiota influence human physiology, metabolism, nutrition, and immune function. The gut contains an estimated 1000 bacterial species and 100-fold more genes than are found in the human genome. Each person has a unique gut microbiota as personal as a fingerprint. It is believed that about 90% of all human diseases can be linked to the microbiome in one way or the other. Still, most of our microbiome is nonculturable.

Metagenomics was developed to identify nonculturable microorganisms; it is the identification of microbial genomes from mixed populations using molecular techniques. Both sequencing and gene expression methods have been used in metagenomics. This method has great application in environmental studies; for example, metagenomics has been applied to study microbial populations from soil, oceans, and biofilms. Metagenomics has also been used to study populations of microorganisms from the human body, such as bowel or urogenital tract microbiota. Many human infections are polymicrobial and involve biofilms (see [Chapter 31](#)). It is possible that many of the bacteria that play a role in infectious processes are not cultivable in the laboratory. Scientists from the J. Craig Venter Institute (JCVI) are traveling the globe by yacht to sample marine microorganisms under a project termed the *Sorcerer II Expedition*. In short, the potential application of metagenomics is limited only by the imagination.

Sherlock microbial identification system

The Sherlock Microbial Identification System (MIDI, Newark, DE) is based on the 9- to 20-carbon fatty acid composition of microorganisms ([Fig. 11.19](#)). The Sherlock system examines which fatty acids are present as well as their relative concentration (percentage). Fatty acids are located in the plasma membrane of bacteria and, depending on environmental conditions, are modified by the bacteria. For this reason, it is important that growth conditions are well standardized for accurate identification.

The standard incubation conditions for most aerobic bacteria are tryptic soy agar incubated at 28°C for 24 hours. After incubation, a loopful of bacteria is suspended in a methanolic base and heated for 30 minutes in a boiling water bath. During this step, cells are lysed, and the fatty acids are cleaved from the lipids and converted to the sodium salt. After cooling, the sample is mixed with a solution of methanol and hydrochloric acid to methylate the fatty acids to fatty acid methyl esters. The fatty acid methyl esters are extracted, washed, and injected into a high-resolution gas chromatograph. The fatty acid esters are separated by the column in the gas chromatograph, and a chromatogram is created. The size of the molecule and degree of saturation determine the retention time in the column. The fatty acids are identified by their retention time compared with standards, and the height of the peak determines the concentration. Typical identification requires 1.5 to 2 hours. The Sherlock system compares the fatty acid



Fig. 11.19 Sherlock Microbial Identification System incorporates Agilent gas chromatograph (#6850). (Courtesy Agilent Technologies, Inc., Palo Alto, CA.)

profile of the unknown with the database of approximately 1500 organisms. MIDI also markets a high-performance liquid chromatography system for the identification of *Mycobacterium* spp. In a study of the identification of environmental airborne bacteria that included *Bacillus* spp., *Staphylococcus* spp., *Micrococcus* spp., and *Acinetobacter*, MIDI correctly classified 75% (77/103) of isolates.

Nanomedicine

Nanotechnology is the creation of functional materials, devices, and systems through the understanding and control of matter at dimensions in the nanometer scale, in which new functionalities and properties of matter are observed and harnessed for a broad range of applications. *Nanomedicine* is defined as the application of nanotechnology in medicine, including diagnosing and treating diseases and repairing damaged tissues such as bone, muscle, and nervous tissue. The advantages of incorporating drugs into nanoparticles are to minimize the side effects of the drug on the body while maximizing the efficacy of the drug. Nanomedicine has the potential to eliminate the risk of side effects by preventing the spread of drugs to undesired locations of the body and may also help develop cost-effective treatments for many chronic diseases. Nanoparticles can be used to deliver antimicrobial agents to the site of infection; see [Chapter 12](#).

Nanotechnology can also provide sensitive and specific diagnosis of infectious diseases by selectively capturing and distinguishing microbial target molecules from other substances in a biological sample. Nanoparticles coupled to probes (e.g., antibodies and nucleic acids), with affinity for microbial biomarkers, can selectively recognize microbes. Nanomaterials used for microbial diagnosis include magnetic, gold, and fluorescent nanoparticles.

Magnetic nanoparticles, such as superparamagnetic iron oxide nanoparticles (SPIONs), have been used as markers to detect nucleic acid sequences of five species of yeasts in the

genus *Candida* in human blood. Hybridization of oligonucleotides labeled with SPIONs to amplified *Candida* DNA produces large changes in the sample's T2 magnetic resonance signal. The assay takes about 3 hours to perform. Gold nanoparticles have also been used as oligonucleotide labels for detecting bacterial DNA sequences, such as the *mecA* gene coding for methicillin resistance in *S. aureus*. Antibody-conjugated silica nanoparticles that encapsulate thousands of fluorescent dye molecules produce an amplified signal that can detect a single bacterial cell in tissue in less than 20 minutes.

POINTS TO REMEMBER

- The three basic nucleic acid molecular diagnostics applications used in clinical microbiology laboratories are (1) nucleic acid hybridization assays, (2) nucleic acid amplification techniques, and (3) strain typing techniques.
- Nucleic acid hybridization assays detect nucleic acid targets with labeled probes. These assays can occur on a solid support, in situ, or in a solution.
- Amplification procedures exponentially increase the amount of target nucleic acid, probe, or signal in the presence of target nucleic acid. Amplification procedures include PCR and variants of PCR (e.g., RT-PCR, multiplex PCR, and nested PCR), NASBA, TMA, bDNA assay, and hybrid capture.
- PCR is frequently used in laboratories and consists of three basic steps: denaturation of target DNA, primer annealing, and primer extension. These steps are repeated multiple times (a cycle) to exponentially increase the amount of target DNA to levels high enough for easy detection.
- Real-time PCR is a popular detection technique used to view PCR products as they are formed in the reaction. The technique is also used to quantitate DNA.
- RT-PCR detects target RNA. The initial step of RT-PCR produces cDNA copies of RNA with reverse transcriptase. The cDNA is then amplified by PCR. RT-PCR, like standard PCR, is often analyzed by real-time PCR.
- Multiplex PCR uses more than one primer set during PCR. One of the primer sets often targets an internal control gene, while the other primer set is used to amplify the target gene.
- Nested PCR is essentially two different PCR assays that are run one after the other. The first assay uses a primer pair that amplifies target DNA. The PCR product is then used as the DNA template for the second PCR assay. The second assay primers are internal to the first PCR product.
- NASBA and TMA are performed at a single temperature (isothermal) and produce large amounts of RNA copies of the target nucleic acid. NASBA uses three enzymes: reverse transcriptase, RNase H, and T7 polymerase. In TMA, a reverse transcriptase with RNase H activity is used along with a T7 RNA polymerase.
- Branched DNA assays use several different probes to amplify the signal instead of the nucleic acid sequence. A branched probe that allows many detection probe molecules to bind to it is used to amplify the signal.
- Hybrid capture uses an RNA probe to bind to target DNA and form an RNA:DNA hybrid. A capture probe is added that binds the hybrid to a solid support. Detection probes are then added that amplify the signal.
- Invader technology uses two oligonucleotides (a primary probe oligonucleotide and an Invader oligonucleotide) that anneal to target DNA. When these two probes anneal to the target DNA, they overlap. The resulting structure is targeted by the enzyme cleavase, which releases a 5' flap that then anneals to a FRET oligonucleotide. This creates a new invasive structure that cleavase acts on. This releases a signal. There is a large excess of probe in the mixture, so many signals are generated if the target sequence is present.
- Strain typing methods are used generally for epidemiologic purposes. Either amplification-dependent or amplification-independent methods are used to detect different strain types. Amplification-independent methods include Southern blotting, plasmid profile analysis, restriction enzyme digestion of chromosomal DNA, PFGE, and MLEE. Amplified methods include RAPD, Rep-PCR, and MLST.
- Of the amplification-independent typing procedures, PFGE is probably used the most by clinical microbiology laboratories. Chromosomal DNA from strains of interest is digested with a restriction enzyme and separated by an electrophoresis system that electrically pulses the large fragments of DNA through an agarose gel. Different strains will have unique banding patterns.
- Among amplification-dependent methods, RAPD-PCR uses random primers that anneal to random sequences in the genome of a given strain. After amplification by PCR, different strains will have unique patterns. Rep-PCR uses primers that anneal to repeating palindromic sequences in a genome. The DNA in between the palindromic sequence repeats is amplified by PCR, resulting in unique patterns for individual strains.
- MLVA amplifies variable numbers of repeats in bacterial genomes.
- MLST identifies mutations in genes by sequencing different loci from strains after PCR amplification. The technique produces excellent intralaboratory and interlaboratory comparisons, although the procedure is expensive and requires sequencing equipment.
- Different sequencing platforms have been developed to identify the sequence of whole organisms or certain regions to yield a highly accurate and sensitive diagnosis.
- Metagenomics allow the identification of nonculturable species using genome sequencing.
- Protein profiles of microorganisms can be determined using MALDI-TOF mass spectrometry. The profiles can be compared with a database for identification of unknown microorganisms.
- Fatty acid profiles of bacteria grown under standard conditions can be detected using gas liquid chromatography and high-performance liquid chromatography. The profiles can be compared with a database to identify the unknown microorganism.
- Nanoparticles, such as magnetic nanoparticles, gold, and fluorochromes, have been used to label probes in the detection of microbial pathogens.

LEARNING ASSESSMENT QUESTIONS

1. Which of the following statements is true regarding Southern blotting?
 - a. It detects transcripts after restriction enzyme digestion and separation by agarose gel electrophoresis.
 - b. It detects a DNA target after restriction enzyme digestion and separation by agarose gel electrophoresis.

- c. It detects protein polymorphisms after separation by electrophoresis.
 - d. It is an amplification technique that analyzes a particular DNA target.
2. Which of the following probe labels is most often used for nucleic acid hybridization reactions in clinical microbiology laboratories?
 - a. Chemiluminescent
 - b. ^{32}P
 - c. ^{35}S
 - d. ^3H
 3. Which of the following is not a component of a standard polymerase chain reaction (PCR) assay?
 - a. Deoxynucleotides
 - b. Primers
 - c. Restriction enzymes
 - d. Magnesium
 4. Which of the following is an advantage of using nucleic acid amplification procedures in clinical microbiology laboratories?
 - a. They are highly sensitive.
 - b. Results are obtained more rapidly compared with standard microbiology techniques such as culture.
 - c. They have increased sensitivity compared with standard microbiology procedures.
 - d. All the above.
 5. Which of the following describes branched DNA detection?
 - a. A form of PCR that analyzes transcripts
 - b. A signal amplification method that uses capture, target, preamplifier, amplifier, and labeled probes
 - c. A signal amplification method that uses RNA probes, capture probes, and alkaline phosphatase-labeled probes
 - d. A target amplification method that uses reverse transcriptase, ribonuclease H (RNase H), and T7 RNA polymerase
 6. Which of the following procedures does not use fluorescence resonance energy transfer (FRET)?
 - a. 5' nuclease assay
 - b. Scorpion primers
 - c. Molecular beacons
 - d. Transcription-mediated amplification
 7. Reverse transcription PCR uses which of the following?
 - a. Reverse transcriptase to produce complementary DNA (cDNA) from transcript
 - b. T7 RNA polymerase to produce transcript from cDNA
 - c. T7 RNA polymerase to produce cDNA from transcript
 - d. RNase H to degrade RNA in DNA:RNA hybrids
 8. Which of the following factors does not influence hybridization reactions?
 - a. pH
 - b. Temperature
 - c. Length of the target's genome
 - d. Degree of complementarity between the probe and target nucleic acid
 9. Which of the following strain typing procedures has probably the greatest interlaboratory agreement?
 - a. Multilocus enzyme electrophoresis
 - b. Multilocus sequence typing
 - c. Pulsed-field gel electrophoresis
 - d. Random amplified polymorphic DNA analysis
 10. Which of the following is not true about the 5' nuclease assay?
 - a. It is also referred to as the *TaqMan assay*.
 - b. It uses a probe with a 5' fluorophore and a 3' quencher that is degraded by the action of DNA polymerase.
 - c. It uses FRET to keep background fluorescence low.
 - d. It uses a hairpin-shaped probe with a fluorophore on the 5' end and a quencher on the 3' end.
 11. For most bacteria, which target is usually sequenced to confirm the identity of problematic isolates?
 - a. *rpoB*
 - b. 16S ribosomal RNA
 - c. Internal transcribed spacer region 1
 - d. External transcribed spacer region 1

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Antibacterial mechanisms of action and bacterial resistance mechanisms

Brandon C. Ellis and Donald C. Lehman

CHAPTER OUTLINE

Antimicrobial targets and mechanisms of action, 252

- Inhibition of bacterial cell wall biosynthesis, 252
- Inhibition of folate synthesis, 258
- Interference with DNA replication, 258
- Interference with DNA transcription, 258
- Interference of mRNA translation, 258
- Combined mechanisms of action, 260

Mechanisms of antimicrobial resistance, 260

- Origins of antimicrobial resistance, 260
- Intrinsic mechanisms of resistance, 261
- Acquired mechanisms of resistance, 263

β -Lactamase inhibitors, 266

Dissemination of Resistance Determinants, 267

Nanotechnology to deliver therapeutic agents, 268

Bibliography, 270

OBJECTIVES

After reading and studying this chapter, you should be able to:

1. Categorize the different classes of antibacterial agents based on mode of action.
2. Contrast the differences between intrinsic and acquired mechanisms of antimicrobial resistance.
3. Discuss how the spectrum of β -lactam antibiotics can be expanded to treat infections caused by β -lactamase-producing bacteria.
4. Discuss how “antibiotic stewardship” can preserve the effectiveness of antimicrobial agents.
5. Describe the mechanisms used by bacteria to exhibit antimicrobial resistance, (e.g., efflux pumps, enzymatic inactivation).
6. Compare intrinsic and acquired antimicrobial drug resistance in bacteria.
7. Correlate the presence of extended-spectrum β -lactamases in gram-negative bacteria with drug resistance.
8. Discuss the mechanisms used by microorganisms to disseminate resistant determinants.
9. Explain how nanomedicine delivery systems can more effectively treat bacterial infections compared with free drugs.
10. Discuss potential alternatives to treat antimicrobial-resistant infections.

KEY TERMS

Acquired resistance

Aminoglycosides

Antibiotic

Antibiotic stewardship

Antimicrobial agent

Avibactam

β -Lactam antibiotics

β -Lactam ring

β -Lactamase inhibitors (BLIs)

β -Lactamases

Biofilms

Clavulanic acid

Conjugation

DNA replication

DNA transcription

Efflux pumps

Glycopeptides

Glycylcyclines

Induced resistance

Insertion sequences (ISs)

Integrons

Intrinsic resistance

Ketolides

Linezolid

Macrolides

mRNA translation

Oxazolidinones

Penicillin-binding proteins (PBPs)

Peptidoglycan

Persister cells

Plasmids

Polymyxin

Porins

Quinolones

Relebactam

Streptogramin

Sulbactam

Sulfamethoxazole (SMZ)

Tazobactam

Tetracycline

Tigecycline

Tolerance

Transcription

Transduction

Transformation

Transposons (Tns)

Trimethoprim (TMP)

Case in point

A 12-year-old male patient had acute abdominal pain with fever. The patient had been well, except for a sinus infection treated with amoxicillin-clavulanate. He finished a 10-day course of treatment 4 days before the onset of abdominal pain. He began a prophylactic regimen of ampicillin-sulbactam and underwent an appendectomy. Treatment with ampicillin-sulbactam was discontinued 2 days after surgery, and treatment with amoxicillin-clavulanate was begun. The following day, the patient exhibited diarrhea, and a stool culture yielded *Salmonella enterica* serotype Typhimurium. The isolate

was reported to be resistant to a wide range of antimicrobial agents, including ampicillin, chloramphenicol, tetracycline, sulfisoxazole, kanamycin, streptomycin, cephalothin (first-generation cephalosporin), and extended-spectrum cephalosporins (e.g., ceftriaxone, ceftazidime, and cefoxitin). An epidemiologic investigation found that the patient's father had treated calves with severe diarrhea from his herd and three other herds 1 month before the patient became ill. Stool specimens from ill cattle in each herd were analyzed and yielded *S. enterica* serotype Typhimurium. Antimicrobial susceptibility testing revealed that the isolate from the patient and one of the isolates from cattle had an identical resistance pattern (antibiogram). Molecular typing by pulsed-field gel electrophoresis showed that the *S. enterica* serotype Typhimurium isolated from the patient and cattle had indistinguishable deoxyribonucleic acid patterns. Further studies were conducted to characterize the mechanisms of resistance and their possible means of transmission.

Issues to consider

After reading the patient's case history, consider:

- The role empiric therapy plays in the treatment of disease
- Whether prior antimicrobial treatment provides insight into possible mechanisms of resistance
- How the bacterial targets of the different drug classes differ
- Which antimicrobial resistance mechanisms can produce the same resistance phenotype
- Which mechanism of resistance is most likely to produce cross-resistance to multiple classes of agents
- The role β -lactamase inhibitors may have played in the treatment of the infection

The discovery of potent, relatively nontoxic antimicrobial therapeutic agents was perhaps the foremost medical advance of the 20th century. **Antimicrobial agents** include antibacterials, antifungals, antiseptics, disinfectants, sterilants, and preservatives; all have the capacity to kill or suppress the growth of microorganisms. Antimicrobial agents are an essential component of the practice of medicine. They treat, prevent, and control the dissemination of microbial pathogens in and on animal tissue and nonliving materials (fomites). The term **antibiotic** has been traditionally reserved for compounds that are naturally produced by living microorganisms, such as bacteria and fungi. The term has come to be more widely applied to any natural, semi-synthetic, or synthetic molecule used to treat or prevent disease. Antimicrobial agents can be natural products, semi-synthetic variants of natural products, or synthetic chemicals designed to block key biochemical pathways or interfere with cellular structures, leading to growth inhibition or bacterial death. Although numerous antimicrobial classes have been discovered, with different modes of action, bacteria evolve and adopt numerous strategies to counteract the action of these agents.

Antimicrobial drug resistance was observed soon after the discovery of antibiotics. It is a natural consequence of drug exposure and results from the use and inappropriate use of antimicrobial agents. The World Health Organization (WHO) estimates that by 2050 there will be at least 10 million deaths per year resulting from infections with drug-resistant bacteria. Besides deaths, it is estimated that drug-resistant

infections could reduce world gross domestic production by 2% to 3% per year, imposing a global economic burden amounting to trillions of dollars. Because of these projections, the WHO has listed antimicrobial resistance as a global public health threat. Antimicrobial use induces selection pressure, allowing only the fittest and most resistant bacterial populations to thrive and thereby displace susceptible populations. Over time and with the use of multiple antimicrobial agents, resistant strains predominate and often acquire multidrug resistance (MDR; also multidrug-resistant). Not only do bacteria acquire the mechanisms necessary to withstand the toxic effects of antimicrobial agents, but they also acquire elaborate mechanisms to mobilize and disseminate genes involved in these successful strategies via plasmids, **transposons (Tns)**, integrons, and other mechanisms (see Chapter 1).

The adaptive strategies used by microorganisms to survive the hostile antimicrobial environment are remarkable in their evolution and complexity. Deoxyribonucleic acid (DNA) sequence data show that many different resistance determinants can amass in linked clusters on plasmids or the chromosome, so antimicrobials of a different class, including substances such as disinfectants or heavy metals, can select for MDR bacteria. Although resistance, particularly MDR, appears to be most serious in certain bacterial species, this situation may be shifting as large, mobile MDR elements spread to new bacterial hosts in different environments.

This chapter introduces the mechanisms of action of antibacterials and how bacteria become resistant to these agents. For information on the action of antifungals and antivirals, see Chapters 27 and 29, respectively. Intrinsic and acquired mechanisms that facilitate resistance to important therapeutic regimens are included. Many of these resistance mechanisms may be generalized to other pathogens. Furthermore, multiple mechanisms of resistance may be present simultaneously in a single microorganism, resulting in the MDR phenotypes frequently observed in clinical settings.

Case check 12.1

The patient in the Case in Point suffered from an infection by an MDR bacterium. The continuous development of new antimicrobial classes of compounds historically has helped medical science stay ahead of developing resistance. However, the number of new antimicrobial agents being brought to the market has undergone a steady decline in the past two decades, making drug resistance and its associated clinical failure an issue of global public health concern. The WHO has published a list of priority pathogens for which new antimicrobials are critically needed. This list contains carbapenem-resistant *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and Enterobacterales.

Only a few new classes of antimicrobial drugs have been approved by the U.S. Food and Drug Administration (FDA) in the past few years: oxazolidinone (2000), lipopeptide (2003), pleuromutilin (2007), and macrolactone (2011). Efforts to develop new analogues of older classes by adding chemical moieties that provide some protection against one or more of the known resistance mechanisms are ongoing. These efforts have resulted in the development of several

well-known classes such as semi-synthetic and synthetic β -lactams, oxazolidinone, ketolides, glycyclines, and glycopeptides. Further exploration of antimicrobial targets, new and old, is vital to the discovery of new antibacterial drugs to address the escalating MDR problem and overcome the loss of effectiveness over time for existing drugs.

To address the issue of MDR infections, programs emphasizing *antibiotic stewardship* have been initiated by health professionals. **Antibiotic stewardship** refers to the appropriate use of antimicrobials to maximize their current effects and improve their chances of being available for future generations. To encourage the development of antimicrobial drugs, new models for promoting their development have been proposed. These models were a focus of the first G20 Health Ministers' Meeting held in May 2017 in Berlin, Germany. Innovative public-private partnerships, such as Combating Antibiotic Resistant Bacteria Biopharmaceutical Accelerator (CARB-X), were formed to assist in the development of new therapeutics, vaccines, diagnostics, and devices to address the issue of MDR infections.

The development of rapid diagnostic tests for use at the patient's bedside (i.e., point of care) to detect the presence of bacterial pathogens and determine their susceptibility to antimicrobials is also essential to reducing the incidence of drug-resistant infections. Diagnostics based on DNA assays such as the polymerase chain reaction and other nucleic acid amplification tests might provide the means to meet the challenge of reducing MDR infections by quickly identifying resistant strains.

In addition, alternatives to the use of antimicrobial drugs are being proposed. Alternatives to antimicrobials are defined as noncompound approaches, products other than classic antibacterial agents, which target bacteria or any approach that targets the host. Currently, the most advanced approaches are antibodies, probiotics, and vaccines. In addition to these therapeutics, the use of bacteriophage therapy as a complement or alternative to traditional antimicrobials is being explored; however, this area is still in the research and development stage.

Antimicrobial targets and mechanisms of action

The most widely used antimicrobial agents interfere with cellular processes and can be grouped into one of six target categories (Table 12.1):

- Bacterial cell wall biosynthesis
- Bacterial cell membranes
- Bacterial protein biosynthesis
- DNA replication and repair
- RNA synthesis
- Folate biosynthesis

Approximately 23 unique classes and 18 subclasses of clinically useful antibacterials representing approximately 100 agents are used in clinical medicine. The classification scheme is complex, and the number of antimicrobials continue to expand as new classes emerge or existing classes are

modified. Antimicrobial agents target anabolic cellular processes such as cell wall synthesis, folate synthesis, DNA replication, ribonucleic acid (RNA) transcription, and messenger RNA (mRNA) translation. These targets are critical to the survival of microorganisms and are sufficiently distinct from eukaryotic cells to permit selective toxicity. Understanding the mechanisms of action of antimicrobial agents provides insight into strategies used by microorganisms to evade their toxic effects. Fig. 12.1 shows the primary cellular process of antibacterial action for major classes of antimicrobial agents (see Table 12.1).

Inhibition of bacterial cell wall biosynthesis

Many antibacterial agents function by targeting bacterial cell wall synthesis. Cell walls are not found in mammalian cells and differ in composition among various bacterial species. Therefore cell wall synthesis provides a number of potential targets for antimicrobial drugs.

Gram-positive and gram-negative bacteria have a multi-layered cell wall structure composed of an inner cytoplasmic membrane, a peptidoglycan layer, and in gram-negative bacteria, a second outer membrane (Fig. 12.2). The cytoplasmic membrane, composed of phospholipids and proteins, surrounds the cytoplasm, acts as an osmotic barrier, and is the location of the electron transport system responsible for energy production.

Peptidoglycan (murein) is a unique mucopolysaccharide constituent of the bacterial cell wall. The quantity of this polymer and its location within the cell envelope are different between gram-negative and gram-positive bacteria. The peptidoglycan layer is made up of chains of alternating disaccharide subunits of *N*-acetyl-D-glucosamine and *N*-acetyl-D-muramic acid (NAM; Fig. 12.3). The NAM subunit has a short peptide chain attached that mediates cross-linking of parallel glycan molecules in mature peptidoglycan. This peptide consists of L- and D-amino acids, which typically end in D-alanyl-D-alanine (D-Ala-D-Ala). Cross-links between neighboring peptide side chains impart mechanical strength to the molecule and present opportunities for biochemical diversity in the types of cross-links within and among different bacterial species. In gram-positive bacteria, the peptidoglycan layer is substantially thicker and more multilayered than in gram-negative bacteria. The outer membrane of gram-negative bacteria is composed of lipopolysaccharides (LPSs), phospholipids, and porin proteins and is separated from the cytoplasmic membrane by the periplasmic space and a thin layer of peptidoglycan.

Peptidoglycan biosynthesis has four major stages: (1) synthesis of precursors in the cytoplasm; (2) transport of lipid-bound precursors across the cytoplasmic membrane; (3) insertion of glycan units into the cell wall; and (4) transpeptidation linking and maturation. D-Cycloserine and bacitracin inhibit the first two steps, respectively. The most used inhibitors of cell wall biosynthesis, β -lactams and glycopeptides, act on processes in stages 3 and 4. Under normal growing conditions, peptidoglycan synthesis proceeds by the ligase-mediated formation of D-Ala-D-Ala, a precursor used to form UDP-NAM-acetyl-muramyl-pentapeptide (see

Table 12.1 Antimicrobial agent targets and pathways

Drug type	Drug name(s)	Derivation	Species range	Primary target	Pathways affected
Fluoroquinolones^a					
DNA synthesis inhibitor	Nalidixic acid, ciprofloxacin, levofloxacin, gemifloxacin, moxifloxacin, norfloxacin	Synthetic	Aerobic gram-positive and gram-negative species, some anaerobic gram-positive species (<i>Clostridium perfringens</i>), and <i>M. tuberculosis</i>	Topoisomerase II (DNA gyrase), topoisomerase IV	DNA replication, SOS response, cell division, ATP generation, TCA cycle, Fe-S cluster synthesis, ROS formation, and envelope and redox-responsive two-component systems
Sulfonamides and trimethoprim					
DNA synthesis inhibitor	TMP/SMX (brand names Bactrim, Septra)	Synthetic	Aerobic gram-positive and gram-negative species	Tetrahydrofolic acid synthesis inhibitors	Nucleotide biosynthesis and DNA replication
Rifamycins					
RNA synthesis inhibitor	Rifampin, rifapentine, rifaximin, rifabutin	Natural and semi-synthetic forms of ansamycins (derived from <i>S. mediterranei</i>)	Gram-positive and gram-negative species, and <i>M. tuberculosis</i>	DNA-dependent RNA polymerase	RNA transcription, DNA replication and SOS response
β-Lactams^a					
Cell wall synthesis inhibitor	Penicillins (penicillin G, penicillin V, amoxicillin, ampicillin, oxacillin, ticarcillin, piperacillin), cephalosporins (cefadroxil, cephalexin, cefprozil, cefazolin, cefdinir, cefiderocol, cefoxitin, cefotaxime, cefotetan, ceftaroline, ceftriaxone, cefuroxime, ceftazidime, cefepime), carbapenems (doripenem, ertapenem, imipenem, meropenem), monobactam (aztreonam)	Natural and semi-synthetic forms of carbonyl-lactam ring-containing azetidinone molecules (from <i>P. notatum</i> , <i>C. acremonium</i> , and <i>S. cattleya</i>)	Aerobic and anaerobic gram-positive and gram-negative species	Penicillin-binding proteins	Cell wall synthesis, cell division, autolysin activity (regulated by LytSR-VncRS two-component system), SOS response, TCA cycle, Fe-S cluster synthesis, ROS formation, and envelope and redox-responsive two-component systems
Glycopeptides and glycolipopeptides					
Cell wall synthesis inhibitor	Vancomycin, teicoplanin, oritavancin, telavancin, dalbavancin	Natural and semi-synthetic forms of amino sugar-linked peptide chains (for glycopeptides) or of fatty acid-bearing, amino sugar-linked peptide chains (for glycolipopeptides) derived from actinobacteria	Gram-positive species	Peptidoglycan units (terminal D-Ala-D-Ala dipeptide)	Cell wall synthesis, transglycosylation, transpeptidation, and autolysin activation (VncRS two-component system)
Lipopeptides					
Cell wall synthesis inhibitor	Daptomycin	Natural and semi-synthetic forms of fatty acid-linked peptide chains (from <i>S. roseosporus</i>)	Gram-positive species (daptomycin), gram-negative species (polymyxins)	Cell membrane	Cell wall synthesis and envelope two-component systems
Oxazolidinones					
Protein synthesis inhibitor	Linezolid, tedizolid	Natural and synthetic	Gram-positive species and <i>M. tuberculosis</i>	50S ribosome	Protein synthesis by preventing ribosome assembly

Continued

Table 12.1 Antimicrobial agent targets and pathways – Cont'd

Drug type	Drug name(s)	Derivation	Species range	Primary target	Pathways affected
Aminoglycosides					
Protein synthesis inhibitor	Gentamicin, kanamycin, neomycin, streptomycin, tobramycin, amikacin	Natural and semi-synthetic forms of amino sugars (-mycins from <i>Streptomyces</i> spp. and -micins from <i>Micromonospora</i> spp.)	Aerobic gram-positive and gram-negative species, and <i>M. tuberculosis</i>	30 S ribosome	Protein translation (mistranslation by tRNA mismatching), ETC, SOS response, TCA cycle, Fe-S cluster synthesis, ROS formation, and envelope and redox-responsive two-component systems
Tetracyclines and glycyl cyclines					
Protein synthesis inhibitor	Tetracycline, doxycycline, minocycline, tigecycline	Natural and semi-synthetic forms of four-ringed polyketides (from <i>S. aureofaciens</i> and <i>S. rimosus</i>)	Aerobic gram-positive and gram-negative species	30 S ribosome	Protein translation (through inhibition of aminoacyl-tRNA binding to ribosome)
Macrolides					
Protein synthesis inhibitor	Erythromycin, clarithromycin, azithromycin	Natural and semi-synthetic forms of 14- and 16-member lactone rings (from <i>S. erythraea</i> and <i>S. ambofaciens</i>)	Aerobic and anaerobic gram-positive and gram-negative species	50 S ribosome	Protein translation (through inhibition of elongation and translocation steps) and free tRNA depletion
Streptogramins					
Protein synthesis inhibitor	Pristinamycin, quinupristin-dalfopristin	Natural and semi-synthetic forms of pristinamycin I (group B, macrolactone ringed-peptides) and pristinamycin II (group A, endolactone oxazole nucleus-bearing depsipeptides) (from <i>Streptomyces</i> spp.)	Aerobic and anaerobic gram-positive and gram-negative species ^b	50 S ribosome	Protein translation (through inhibition of initiation, elongation, and translocation steps) and free tRNA depletion
Phenicol					
Protein synthesis inhibitor	Chloramphenicol	Natural and semi-synthetic forms of dichloroacetic acid with an aromatic nucleus and aminopropanediol chain (from <i>S. venezuelae</i>)	Some gram-positive and gram-negative species, including <i>B. fragilis</i> , <i>N. meningitidis</i> , <i>H. influenzae</i> , and <i>S. pneumoniae</i>	50 S ribosome	Protein translation (through inhibition of elongation step)
Polypeptides					
Disrupts bacterial cell membrane	Polymyxin B, polymyxin E (colistin)	Contain non-protein polypeptide chains, originally from <i>B. polymyxa</i>	Only against gram-negative bacilli, particularly <i>Pseudomonas</i> spp. and <i>Acinetobacter</i> spp.	Phospholipids in cell membrane	Increases cell permeability and disrupts osmotic integrity
Lincosamides					
Protein synthesis inhibitor	Lincomycin, clindamycin	Natural and semi-synthetic forms of lincomycin originally from <i>S. lincolnensis</i>	Clindamycin broad spectrum against anaerobic gram-positive species	50 S ribosome	Prevents elongation of peptide chains by interfering with peptidyl transfer

^aDrug efficacy can vary across species range based on drug generation.

^bWhen used as a combination of pristinamycin I and pristinamycin II.

B. fragilis, *Bacillus fragilis*; *B. polymyxa*, *Bacillus polymyxa*; *C. acremonium*, *Cephalosporium acremonium*; *C. perfringens*, *Clostridium perfringens*; ETC, electron transport chain; Fe-S, iron-sulfur; *H. influenzae*, *Haemophilus influenzae*; *M. tuberculosis*, *Mycobacterium tuberculosis*; *N. meningitidis*, *Neisseria meningitidis*; *P. notatum*, *Penicillium notatum*; ROS, reactive oxygen species; *S. ambofaciens*, *Streptomyces ambofaciens*; *S. aureofaciens*, *Streptomyces aureofaciens*; *S. cattleya*, *Streptomyces cattleya*; *S. erythraea*, *Saccharopolyspora erythraea*; *S. lincolnensis*, *Streptomyces lincolnensis*; *S. mediterranei*, *Streptomyces mediterranei*; *S. pneumoniae*, *Streptococcus pneumoniae*; *S. rimosus*, *Streptomyces rimosus*; *S. roseosporus*, *Streptomyces roseosporus*; *S. venezuelae*, *Streptomyces venezuelae*; TCA, tricarboxylic acid; TMP/SMX, trimethoprim/sulfamethoxazole; TCA cycle, the citric acid cycle; tRNA, transfer RNA.

Modified from Kohanski, M. A., et al. (2010). How antibiotics kill bacteria: from targets to networks. *Nature Reviews Microbiology*, 8, 423.

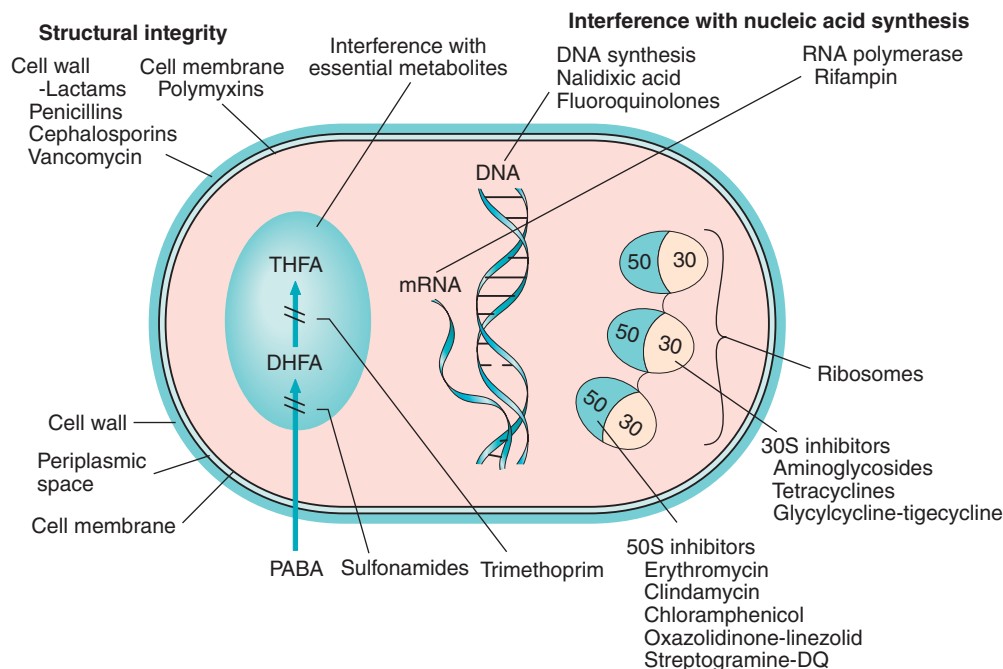


Fig. 12.1 Primary sites of antibacterial action for major classes of antimicrobial agents. *DHFA*, Dihydrofolic acid; *DNA*, deoxyribonucleic acid; *mRNA*, messenger RNA; *PABA*, *p*-aminobenzoic acid; *THFA*, tetrahydrofolic acid.

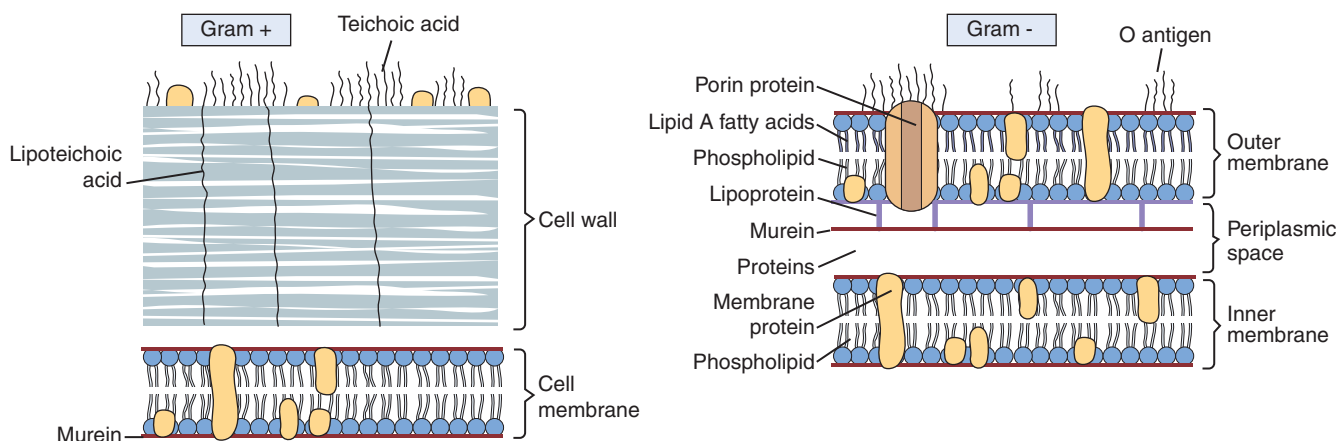


Fig. 12.2 The cell envelope structure of a gram-positive (*left*) and a gram-negative (*right*) bacterium.

Fig. 12.3). This precursor molecule elongates peptidoglycan by performing transglycosylation of the glycan strands; elongation of the peptide strands occurs by transpeptidation.

The **β -lactam antibiotics** include the penicillins, cephalosporins, penems, cephems, carbapenems, and monobactams. These closely related compounds act by forming covalent complexes with enzymes that generate the mature peptidoglycan molecule. Because the functions of these enzymes were studied in the context of penicillin binding and resistance, they are known collectively as **penicillin-binding proteins (PBPs)**. PBPs are bifunctional transpeptidase-transglycosylase enzymes that mediate peptidoglycan cross-linking (**Table 12.2**).

The active moiety of β -lactam is the four-member **β -lactam ring** (**Fig. 12.4**). This four-member ring functions as a structural analogue of the normal substrate acyl-D-Ala-D-Ala.

The structural similarity between β -lactam antibiotics and D-Ala-D-Ala facilitates their binding to the active site of PBPs and inhibition of the transpeptidation reaction, resulting in bacterial lysis and cell death. The effects of drug binding on cell growth differ, depending on the agent and PBP involved. Some inhibit cell division, leading to long filamentous forms, whereas others lead to the formation of cell wall-deficient types that readily lyse under high osmotic pressure. In gram-negative cells, the β -lactams must pass through outer membrane porin channels to reach the target PBPs.

The narrow- to broad-spectrum antimicrobial activity, safety, and efficacy of β -lactam antibiotics are typically enhanced by the modification of moieties attached to the penicillin and cephalosporin ring structures (see **Fig. 12.4**). Carbapenems have the broadest spectrum of activity within

the β -lactam class, have a high affinity for PBPs, and are stable against most Ambler class A, C, and D β -lactamases. Doripenem, a carbapenem used for the treatment of complicated urinary tract and intraabdominal infections, is unique in that it has activity against gram-positive and gram-negative bacteria, including *P. aeruginosa*. Although older cephalosporins do not inhibit the PBPs of *P. aeruginosa*, newer ones such as ceftolozane/tazobactam (trade name Zerbaxa) inhibit PBP1b, PBP1c, and PBP3 of *P. aeruginosa*.

Table 12.3 presents a different β -lactamase classification than the Ambler classification that is based on the functional properties of enzymes (i.e., the substrate and inhibitor profiles). This scheme is referred to as the

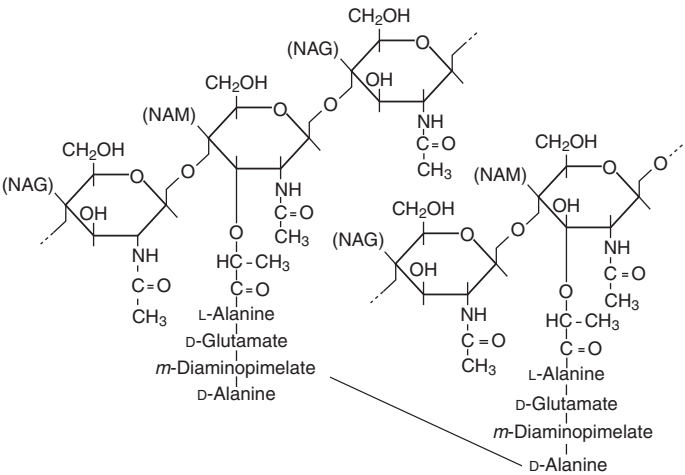


Fig. 12.3 The structure of the peptidoglycan layer in the cell wall of *Escherichia coli*. The amino acids in the cross-linking tetrapeptides may differ among species. NAG, N-acetyl-D-glucosamine; NAM, N-acetyl-D-muramic acid. (From Neidhardt, F. C., et al. [1990]. *Physiology of the bacterial cell: a molecular approach*. Sunderland, MA: Sinauer Associates.)

Bush-Jacoby-Medeiros classification. These schemes are for convenience, and one classification is no more correct than the other.

Glycopeptides, such as vancomycin, dalbavancin, teicoplanin, and the investigational drugs oritavancin and telavancin, act by binding to the terminal D-Ala-D-Ala of the pentapeptidyl-glycosyl peptidoglycan intermediates (see Fig. 12.3). This prevents their incorporation into the peptidoglycan chain by blocking the transpeptidation step in cell wall biosynthesis. Glycopeptides bind to the substrate of the transpeptidation enzyme, whereas penicillins bind to the enzyme mediating the transpeptidation reaction. Because they cannot cross the outer membrane of gram-negative bacteria, the clinical spectrum of glycopeptides is limited to gram-positive microorganisms; thus they are mainly used in the United States to treat aerobic clinical infections caused by staphylococci, streptococci, and enterococci.

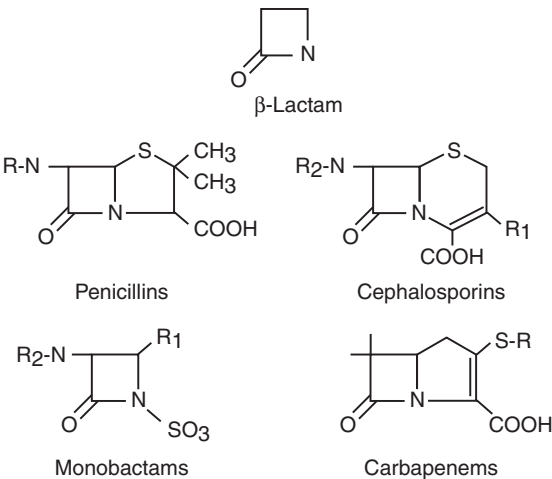


Fig. 12.4 Chemical structures of major classes of β -lactam antibiotics.

Table 12.2 Penicillins, cepheids, carbapenems, and monobactam β -lactam antibiotics			
Class	Subclass	Category	Adopted name in the United States
Penicillins		Narrow spectrum and penicillinase—sensitive	Penicillin G, penicillin V
		Penicillinase—sensitive and semi-synthetic	Amoxicillin, ampicillin
		Penicillinase—resistant and semi-synthetic	Nafcillin, oxacillin
	Aminopenicillin	Extended spectrum and semi-synthetic	Amoxicillin, ampicillin
	Carboxypenicillin	Extended spectrum and semi-synthetic	Ticarcillin, carbenicillin
Cepheids	Ureidopenicillin	Extended spectrum and semi-synthetic	Piperacillin
	Cephalosporin I	Narrow-spectrum, first generation	Cephalothin, cefazolin, cephalexin
	Cephalosporin II	Expanded-spectrum, second generation	Cefonicid, cefuroxime,
	Cephalosporin III	Broad-spectrum, third generation	Cefoperazone, ceftazidime
	Cephalosporin IV	Extended-spectrum, fourth generation	Cefepime, ceftoxitin
	Carbacephem	Broad spectrum	Loracarbef
Carbapenems		Broad spectrum	Doripenem, ertapenem, imipenem, meropenem
Monobactams		Narrow-spectrum	Aztreonam

Table 12.3 Current major β -lactamase functional and molecular classification schemes

Bush-Jacoby group (2010)	Bush-Jacoby-Medeiros group (1995)	Molecular class (subclass)	Distinctive substrate(s)	Inhibited by		Defining characteristic(s)	Representative enzyme(s)
				CA or TZB	EDTA		
1	1	C	Cephalosporins	No	No	Greater hydrolysis of cephalosporins than benzylpenicillin; hydrolyzes cephamycins	<i>E. coli</i> AmpC, P99, ACT-1, CMY-2, FOX-1, MIR-1
1e	NI	C	Cephalosporins	No	No	Increased hydrolysis of ceftazidime and often other oxyimino- β -lactams	GC1, CMY-37
2a	2a	A	Penicillins	Yes	No	Greater hydrolysis of benzylpenicillin than cephalosporins	PC1
2b	2b	A	Penicillins, early cephalosporins	Yes	No	Similar hydrolysis of benzylpenicillin and cephalosporins	TEM-1, TEM-2, SHV-1
2be	2be	A	Extended-spectrum cephalosporins, monobactams	Yes	No	Increased hydrolysis of oxyimino- β -lactams (cefotaxime, ceftazidime, ceftriaxone, cefepime, aztreonam)	TEM-3, SHV-2, CTX-M-15, PER-1, VEB-1
2br	2br	A	Penicillins	No	No	Resistance to CA, sulbactam, and TZB	TEM-30, SHV-10
2ber	NI	A	Extended-spectrum cephalosporins, monobactams	No	No	Increased hydrolysis of oxyimino- β -lactams combined with resistance to CA, sulbactam, and TZB	TEM-50
2c	2c	A	Carbenicillin	Yes	No	Increased hydrolysis of carbenicillin	PSE-1, CARB-3
2ce	NI	A	Carbenicillin, cefepime	Yes	No	Increased hydrolysis of carbenicillin, cefepime, and cefpirome	RTG-4
2d	2d	D	Cloxacillin	Variable	No	Increased hydrolysis of cloxacillin or oxacillin	OXA-1, OXA-10
2de	NI	D	Extended-spectrum cephalosporins	Variable	No	Hydrolyzes cloxacillin or oxacillin and oxyimino- β -lactams	OXA-11, OXA-15
2df	NI	D	Carbapenems	Variable	No	Hydrolyzes cloxacillin or oxacillin and carbapenems	OXA-23, OXA-48
2e	2e	A	Extended-spectrum cephalosporins	Yes	No	Hydrolyzes cephalosporins; inhibited by CA but not aztreonam	CepA
2f	2f	A	Carbapenems	Variable	No	Increased hydrolysis of carbapenems, oxyimino- β -lactams, cephamycins	KPC-2, IMI-1, SME-1
3a	3	B (B1)	Carbapenems	No	Yes	Broad-spectrum hydrolysis, including carbapenems but not monobactams	IMP-1, VIM-1, CcrA, IND-1
		B (B3)					L1, CAU-1, GOB-1, FEZ-1
3b	3	B (B2)	Carbapenems	No	Yes	Preferential hydrolysis of carbapenems	CphA, Sfh-1
NI	4	Unknown					

CA, Clavulanic acid; EDTA, ethylenediaminetetraacetic acid; NI, not included; TZB, tazobactam.

From Bush, K., & Jacoby, G. A. (2010). Updated functional classification of beta-lactamases. *Antimicrobial Agents and Chemotherapy*, 54(3):969–976; and Bush, K., Jacoby, G. A., & Medeiros, A. A. (1995). A functional classification scheme for β -lactamases and its correlation with molecular structure. *Antimicrobial Agents and Chemotherapy*, 39:1211–1233.

Inhibition of folate synthesis

The folic acid pathway provides the essential precursor molecules needed for DNA biosynthesis in bacteria. The pathway is mediated by two key enzymes, dihydropteroate synthase and dihydrofolate reductase, which mediate the formation of tetrahydrofolate (THF) from dihydrofolate (Fig. 12.5). **Sulfamethoxazole** (SMZ) blocks the step leading to the formation of 7,8-dihydropteroate by competitively inhibiting the binding of the structural analogue aminobenzoic acid with dihydropteroate synthase. **Trimethoprim** (TMP) blocks the step leading to the formation of THF by preventing the dihydrofolate reductase-mediated recycling of folate coenzymes. Unlike other members of the current antimicrobial classes, SMZ and TMP are completely synthetic molecules that have never existed in nature. The spectrum of activity of folate pathway inhibitors, especially when provided in combination, provides activity against the Enterobacterales that cause urinary tract infections. TMP and SMZ are often used together under the brand names Bactrim and Septra.

Interference with DNA replication

The prokaryotic cell cycle consists of **DNA replication** followed immediately by cell division. In microorganisms such as *Escherichia coli*, which divide in approximately 30 minutes under ideal growth conditions, DNA replication must be initiated and completed to ensure that each DNA duplex is delivered to each daughter cell. Enzymes necessary for DNA replication include topoisomerases I, II, III, and IV. **Quinolones** are antibacterial drugs that affect DNA

replication by targeting topoisomerases II (DNA gyrase) and IV, enzymes considered important in controlling DNA topology, replication, and decatenation at the end of the bacterial DNA replicative cycle.

DNA gyrase and topoisomerase IV are tetrameric molecules composed of dimeric A and B subunits. The subunits of DNA gyrase are encoded by *gyrA* and *gyrB*, whereas the subunits of topoisomerase IV are encoded by *parC* and *parE*. The tetramers of DNA gyrase and topoisomerase IV are highly homologous, with *gyrA* homologous to *parC* and *gyrB* homologous to *parE*. Interestingly, the targets of quinolones appear to be selective, targeting DNA gyrase in gram-negative bacteria and topoisomerase IV in gram-positive bacteria, but newer quinolones appear to have a high affinity for both targets. Analyses of mechanisms of action suggest that quinolones interact with DNA gyrase–DNA complexes and topoisomerase IV–DNA complexes to trap the enzymes as stabilized reaction intermediates, leading to inhibition of DNA replication, which immediately leads to bacteriostasis and eventual cell death.

The quinolones and fluoroquinolones are used to treat the Enterobacterales, pseudomonads, *Neisseria*, and other gram-negative bacteria as well as staphylococci, enterococci, and streptococcal species other than *Streptococcus pneumoniae*. More so than some other drug classes, the rate of fluoroquinolone resistance has been increasing. Fluoroquinolone resistance correlates with the use of the agent in the human population and is mainly caused by chromosomal mutations.

Interference with DNA transcription

DNA transcription is the process whereby a template DNA strand is copied into a functional RNA sequence, resulting in mature mRNA or structural RNA. The **transcription** of DNA into RNA is mediated by RNA polymerase; bacterial RNA polymerase is a core tetramer composed of an α subunit, two β subunits ($\beta\beta'$), a γ subunit, and a dissociable σ subunit that controls the transcription of specific gene classes.

Rifampin, a synthetic derivative of rifamycin B, targets DNA transcription. The principle therapeutic use of rifampin is in combination with other antibacterial classes to treat *Mycobacterium tuberculosis* infection. The target of rifampin in *M. tuberculosis* is the RNA polymerase β subunit at an allosteric site, with the subsequent blocking of RNA chain elongation. As a result, RNA transcription is aborted at the initiation step. Less commonly, rifampin is used to treat aerobic species including staphylococci, enterococci, *Haemophilus* spp., and *S. pneumoniae*. Rifamycins in combination with ciprofloxacin and clindamycin are reported to be useful for the treatment of bacterial infections relevant to biowarfare or bioterrorism, such as inhalational anthrax.

Interference of mRNA translation

The cellular machinery of living organisms decodes mRNA into functional protein, a process called **mRNA translation**. Protein biosynthesis requires the sequential binding of the 30S and 50S ribosomal subunits to mRNA, leading to translation of the genetic message. The initiation phase commences with initiation factors, proteins that bind to the 30S subunit, and the initiator transfer RNA (tRNA), formylmethionyl-tRNA, which binds to the P site of the 30S ribosomal subunit.

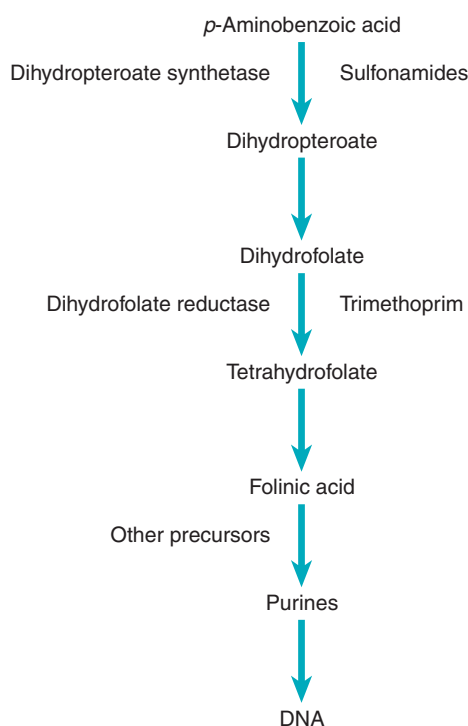


Fig. 12.5 Sites of action of sulfonamides and trimethoprim and their effects on the synthesis of essential amino acids and nucleic acids.

This 30S subunit helps the bound initiator tRNA scan and find the start codon on mRNA. Next, the 50S subunit binds to form the preinitiation complex. The codon immediately following the initiation codon dictates binding of the next tRNA to the ribosomal A site.

Because protein synthesis is central to cellular function and bacterial ribosomes differ from eucaryote ribosomes, it is an excellent target for antibacterial drug product development. Thus the bacterial ribosome is a primary target of numerous drugs, with some targeting the 30S ribosomal subunit (e.g., aminoglycosides, tetracyclines, glycylcyclines) and others targeting the 50S ribosomal subunit (e.g., macrolides, lincosamides, chloramphenicol, oxazolidinones, streptogramins; see Fig. 12.1). Among ribosome inhibitors, naturally derived aminoglycosides are the only class that is broadly bactericidal. Macrolides, streptogramins, spectinomycin, tetracyclines, and chloramphenicol are typically bacteriostatic; however, they can be bactericidal in a species-specific manner (e.g., chloramphenicol has been shown to kill *S. pneumoniae*). This species-specific bactericidal effect probably involves sequence differences among bacterial species in the variable regions of the highly conserved ribosomal RNA (rRNA).

Aminoglycosides are cationic carbohydrate-containing molecules, and their positive charge provides the basis for their interaction with a specific region of the 16S RNA in the 30S ribosomal subunit. The 30S subunit provides a high-affinity docking site mediated by hydrogen bonding to the various substituents in the aminoglycoside cyclitol ring. Binding of aminoglycosides to the A binding site on the 30S subunit prevents the docking of aminoacyl-tRNA, resulting in mistranslation and subsequent production of aberrant proteins. The incorporation of aberrant proteins into the cell wall also results in cell leakage and enhanced cellular penetration of additional antimicrobial agents. To penetrate the bacterial inner membrane and reach the target, an aerobic energy-yielding step is necessary. The requirement for an aerobic environment for activation explains the aminoglycosides' lack of activity against anaerobic bacteria.

Spectinomycin is a disaccharide aminocyclitol antibiotic that is produced by *Streptomyces spectabilis* and is active against many gram-negative bacterial species. It binds to the 16S rRNA component of the 30S ribosomal subunit and interferes with the binding stability of peptidyl-tRNA to the ribosome by inhibiting elongation factor-catalyzed translocation, but it does not cause protein mistranslation.

Tetracycline compounds are members of the polyketide class of antibacterials and are represented by tetracycline, doxycycline, and minocycline. As shown in Fig. 12.1, the tetracyclines target the 30S ribosomal subunit. Tetracyclines reversibly inhibit protein synthesis by binding to 16S rRNA near the aminoacyl-tRNA acceptor site, thus inhibiting the rotation of bound tRNA into the A site during translation. This physical blocking by tetracycline results in the premature release of tRNA and termination of peptide bond formation. The tetracyclines are broad-spectrum, bacteriostatic drugs.

Macrolides, such as erythromycin, clarithromycin, and azithromycin, target the 50S subunit specifically by binding to the peptidyltransferase cavity in the proximity of the A and P loops, near adenine 2058 of 23S rRNA. By blocking the exit tunnel of the elongating peptides, premature release of peptidyl-tRNA intermediates occurs, and polypeptide translation ceases. Macrolides also prevent assembly of the

50S ribosomal subunit by binding to 23S rRNA. As shown in Fig. 12.1, lincosamides (clindamycin) and chloramphenicol also target the 50S ribosomal subunit, thus inhibiting mRNA translation and, subsequently, protein synthesis. Macrolides and tetracyclines allow initiation and mRNA translation to begin but act by inhibiting peptide elongation.

Ketolides share many of the characteristics of the advanced macrolides. They are semi-synthetic derivatives of 14-member-ring macrolides and have a carbonyl group at the C-3 position, which allows them to be active against macrolide-resistant strains, increasing their activity against gram-positive cocci. Telithromycin was the first member of this class to be approved by the FDA for clinical use for the treatment of community-acquired pneumonia. The mechanism of action is very similar to that of erythromycin. The ketolides bind close to the peptidyltransferase site of the 50S subunit of the bacterial ribosome, inhibiting RNA-dependent protein synthesis. Ketolides bypass macrolide-resistant mechanisms by increasing ribosome binding affinity, especially binding to domain II of the 23S rRNA, and evading macrolide efflux mechanisms.

The most recently approved classes of antimicrobial agents targeting protein synthesis are the oxazolidinones, represented by linezolid; the streptogramins, represented by quinupristin-dalfopristin; and the glycylcyclines, represented by tigecycline.

Oxazolidinones have activity against gram-positive bacteria, such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE), *S. pneumoniae*, and *M. tuberculosis*. **Linezolid** is thought to bind to the 50S ribosomal subunit, preventing formation of the preinitiation complex with the 30S ribosomal subunit containing bound initiation factors. The 50S ribosomal target appears to be the ribosomal P site in the peptidyltransferase center, and by blocking this site, the first peptide-forming step is prevented, and protein synthesis is terminated. Linezolid differs from other protein synthesis inhibitors by blocking the initiation step and translocation of peptidyl-tRNA from the A site to the P site, whereas macrolides and tetracyclines block peptide chain elongation.

Glycylcyclines, represented by tigecycline, are derivatives of the tetracycline class and carry a glycylamido moiety attached to the 9-position of minocycline. **Tigecycline** reversibly inhibits protein translation in bacteria by binding to the 30S ribosomal subunit and blocking entry of aminoacyl-tRNA molecules onto the A site of the ribosome, thus preventing the incorporation of amino acid residues into elongating peptide chains. Tigecycline has a stronger binding affinity for the 30S ribosomal subunit than tetracycline and has more potent activity against tetracycline-resistant organisms with efflux and ribosomal protection mechanisms of resistance. Tigecycline has a wide spectrum of activity against many gram-positive and gram-negative organisms as well as some mycobacteria and anaerobic pathogens.

Streptogramin antibiotics are composed of a mixture of two classes of distinct molecules designated *streptogramin A* and *streptogramin B*. Dalfopristin-quinupristin is a combination of these two streptogramins in a ratio of 70:30. Dalfopristin is a polyunsaturated macrolactone classified as a type A streptogramin, and quinupristin is a peptide macrolactone classified as a type B streptogramin. Streptogramins disrupt translation of mRNA into protein by binding to the peptidyltransferase domain of the bacterial ribosome. Dalfopristin interferes with the elongation

of the polypeptide chain by preventing binding of aminoacyl-tRNA to the ribosome and the formation of peptide bonds. Quinupristin stimulates the dissociation of the peptidyl-tRNA and is thought to interfere with the release of the completed polypeptide by blocking the exit tunnel through which it normally leaves the ribosome. Dalfopristin and quinupristin act synergistically because of the enhanced affinity of quinupristin for the ribosome. Dalfopristin induces a conformational change such that quinupristin binds with greater affinity. The natural streptogramins are produced as mixtures of dalfopristin and quinupristin; their combination is a more potent antibacterial agent than either compound alone.

Combined mechanisms of action

Although antibacterial agents may have several mechanisms of action, one mechanism generally predominates. However, newer antimicrobials have several main mechanisms of action. Such an agent is oritavancin, a semi-synthetic lipoglycopeptide with three main mechanisms of action: (1) inhibition of the transglycosylation (polymerization) step of cell wall biosynthesis, (2) inhibition of the transpeptidation (cross-linking) step of cell wall biosynthesis, and (3) disruption of bacterial membrane integrity. All or any one of these mechanisms can be bacteriocidal. Structurally, oritavancin resembles vancomycin and is most active against gram-positive bacteria.

Mechanisms of antimicrobial resistance

Bacteria use two strategies to avoid being killed by antimicrobial agents: *tolerance* and *resistance*. Tolerance is a property of dormant, nongrowing bacteria (persisters) in which drug targets are inactive, allowing bacteria to avoid damage and survive. **Persister cells** are subpopulations of bacteria deep in a biofilm (discussed later) that can differentiate into a phenotypically resistant state (e.g., slow-growing or nongrowing bacteria). Mechanisms of persister formation include a decrease in adenosine triphosphate (ATP) concentration and toxins that stop essential cellular processes. Cells also contain anti-toxins that can block the action of the toxin allowing growth to resume. Persister bacteria have been shown to carry mutations in promoter genes. When conditions are favorable for growth (e.g., no antimicrobial is present), these bacteria will resume growth. Generally, the persister bacteria that resume growth are resistant to the drug to which they were originally exposed because of mutations that have occurred.

Resistance to antimicrobial agents is conveniently divided into mechanisms that are **intrinsic** or acquired resistance. Intrinsic mechanisms of resistance are an innate characteristic of the microorganism and are transmitted to progeny vertically. Such resistance is considered a natural and consistently inherited characteristic of a particular microbial group, genus, or species. Therefore this resistance is predictable once the organism is identified. For example, some gram-negative bacteria are intrinsically resistant to the activity of macrolides because these agents are too large to traverse the cell wall (impermeability).

Acquired mechanisms of resistance are caused by changes in the usual genetic makeup of a microorganism, leading to altered cellular physiology and structure. Unlike intrinsic resistance, acquired resistance can be a trait associated with only some strains of a particular species. Thus the presence of this type of resistance in any of the isolates is unpredictable. The gene changes or exchanges that result from acquired resistance are usually caused by genetic mutation(s), acquisition of genes from other organisms via gene transfer mechanisms, or a combination of mutational and gene transfer events. Where feasible, this section will use members of the antimicrobial classes discussed earlier to exemplify intrinsic or acquired mechanisms of resistance.

Case check 12.2

The discovery of antibacterial agents was a turning point in human history. Antimicrobials have revolutionized medicine in many respects and saved millions of lives. Unfortunately, antimicrobial resistance is an outcome of natural selection, and the use of these drugs has been accompanied by the rapid development of resistance. Currently, over 100 antimicrobials have been approved for use in clinical medicine; however, resistance has developed to each drug, with different times and frequencies, in various bacterial genera. The MDR bacteria causing the infection in the Case in Point likely underwent natural selection because of repeated exposure to numerous antimicrobial agents.

The most common resistance mechanisms bacteria use include enzymatic degradation or alteration of the drug, mutation in the antimicrobial target site, decreased cell wall permeability to antimicrobials, and active efflux of the antimicrobial across the cell membrane. Inappropriate use of antimicrobials in human and veterinary medicine, animal husbandry, and agriculture over many years has resulted in unremitting selection pressure to accelerate resistance development in microbial populations.

Antimicrobial resistance is a significant public health concern. Resistance to commonly used antimicrobials is now encountered frequently, and the emergence of MDR bacteria poses greater challenges to the treatment of infections. Infection with MDR bacteria can cause treatment failure, increase the burden of illness, and result in higher mortality as well as financial burden.

Origins of antimicrobial resistance

Resistance genes and transfer mechanisms existed long before the use of modern therapeutic antimicrobials. For example, MDR bacteria estimated at being more than 2000 years of age were recovered from glacial samples obtained from the Canadian Arctic Archipelago, whereas another study detected β -lactamases from a metagenomic library of cold-seep sediments of deep-sea Edison Seamount (near Papua New Guinea) that were estimated to be about 10,000 years of age. In addition, plasmids found in gram-negative bacteria that were isolated before antimicrobials were introduced into clinical practice resemble currently described plasmids, except that the early isolates did not possess any resistance genes. **Plasmids** are small circular DNA fragments found outside the chromosome.

Resistance to natural antimicrobial agents, synthetic derivatives, and completely synthetic antimicrobials was observed in a collection of soil-dwelling actinomycetes, with some displaying resistance mechanisms not traditionally observed in clinical bacterial pathogens. Most soil isolates display resistance to at least 6 different antimicrobial agents and, in some cases, as many as 20. The use of novel resistance mechanisms by these organisms, coupled with the fact that these soil microbes are not as intensively exposed to antimicrobial selective pressures as the clinical pathogens, emphasize that resistance is a natural part of microbial ecosystems and highlights the evolutionary possibilities for novel antimicrobial resistance determinants.

If bacterial resistance to antimicrobial agents is not a new phenomenon, where did it originate? One popular theory is that antimicrobial resistance mechanisms arose within antibiotic-producing microorganisms as a mechanism to protect them against the action of their own antibiotic (autotoxicity). This has been substantiated by the finding of aminoglycoside-modifying enzymes in aminoglycoside-producing organisms that display marked homology to modifying enzymes found in aminoglycoside-resistant bacteria. Also, the essential genetic determinants associated with resistance to vancomycin, *vanA*, *vanH*, and *vanX*, appear to be very similar to the self-protection mechanism used in the vancomycin-producing *Actinomyces* strains. In addition, some antibiotic preparations used for humans and animals were found to be contaminated with chromosomal DNA of an antibiotic-producing organism, including antimicrobial resistance gene sequences. It has been postulated that the presence of DNA encoding antimicrobial resistance in antibiotic preparations has been a factor in the rapid development of multiple antibiotic resistance by providing the resistance sequences that can be incorporated by the pathogen.

Intrinsic mechanisms of resistance

Intrinsic resistance is the innate ability of a bacterial species to resist the activity of a particular antimicrobial agent through inherent structural or functional characteristics, allowing tolerance of a particular drug or antimicrobial class. Such natural resistance can be caused by (1) lack of affinity of the drug for the bacterial target, (2) inability of the drug to enter the bacterial cell, (3) removal of the drug by chromosomally encoded efflux pumps (constitutive drug efflux), and (4) innate production of enzymes that inactivate the drug. Bacteria differ widely in cell wall composition, thus their intrinsic susceptibility or resistance to antimicrobial agents depends on the hydrophobic or hydrophilic nature of the drug and on the impermeability of the cell wall to the drug. Intrinsic resistance mediated by impermeability is exemplified by cell wall composition, efflux, and biofilm (see Chapter 31) formation.

Biofilms

Biofilms are sessile communities of microorganisms that are irreversibly attached to a solid surface and are embedded in an exopolysaccharide matrix. Biofilms play an important role in animal infections, and they are a cause for concern because the bacteria in biofilms are highly resistant to antimicrobial agents and host defenses. The mechanisms of biofilm-associated

antimicrobial resistance are multifactorial, involving a complex system of cell-to-cell chemical communication that varies from organism to organism. Thus resistance exhibited by bacteria when they grow in biofilms is not attributed to typically acquired genetic mechanisms (e.g., gene mutation, mobile acquisition), but is instead determined by the chemical and physical characteristics of biofilm formation.

The genetic mechanisms of biofilm antimicrobial resistance appear to fall into two general classes: innate resistance factors and induced resistance factors. Innate mechanisms are activated as part of the biofilm developmental pathway; these factors are integral parts of biofilm structure and physiology (e.g., decreased penetration of antimicrobial agents into the biofilm, decreased oxygen and nutrient availability accompanied by altered metabolic activity, formation of persister cells).

Induced resistance factors include those resulting from induction by the antimicrobial agent itself, resulting in differential resistance gene expression throughout the biofilm community. Biofilm antimicrobial resistance is a complex combination of innate anabolic metabolism and induced genetic mechanisms, many of which still need to be elucidated. Because of the important nature of biofilm-associated antimicrobial resistance, numerous investigators are focusing on the development of novel therapies aimed at disrupting biofilms.

Impermeability

For antimicrobials to affect internal cellular processes, they must penetrate the bacterial cell wall to reach their target. Influx of agents through the cell wall depends on the chemical nature of the agent and the structural characteristics of the cell wall (see Fig. 12.2). The intrinsic resistance of gram-negative bacteria to vancomycin is an example of their outer membrane being impermeable to the large, rigid, and hydrophobic glycopeptide molecule vancomycin. Unlike gram-negative bacteria, some gram-positive bacterial species such as *Lactobacillus* and *Leuconostoc* spp. also have intrinsic resistance to vancomycin, but this resistance is caused by the lack of an appropriate cell wall precursor target to allow vancomycin to bind and inhibit cell wall synthesis. *Enterococcus faecalis* and *Enterococcus faecium* are intrinsically resistant to low levels of penicillin because of PBPs (PBP5) with low affinity for penicillins.

In gram-negative bacteria, there are two fundamental structures of the cell wall leading to clinically relevant resistance caused by impermeability: (1) LPS composition and (2) the structure and expression of outer membrane proteins (Omps), called **porins**. Molecular analysis of gram-negative bacteria reveals that the strongly negatively charged core region of the LPS functions as a selective permeability barrier for negatively charged antimicrobial agents, resulting in decreased susceptibility to these compounds. Mutations in the O-antigen side chains of the LPS can change the shape and overall charge of the cell wall, decreasing the binding efficiency of some cationic agents. Mutations in the LPS genes have been seen in conjunction with increasing drug selective pressure. Thus, the cell wall partially accounts for the intrinsic resistance of bacteria to antimicrobial agents. It is usually only clinically significant in the context of other resistance mechanisms, such as efflux (discussed later), that work synergistically to mediate survival of the organism. For example, *P. aeruginosa* is intrinsically resistant to a wide variety of

antimicrobial agents, including β -lactams, β -lactam inhibitors, sulfonamides, trimethoprim, tetracycline, and chloramphenicol. Although this resistance has usually been attributable to a highly impermeable outer membrane, it has also been shown to result from the synergy between a unique efflux system (*MexAB-OprM*) and decreased outer membrane permeability.

Porins serve as natural outer membrane channels that permit the influx of nutrients and efflux of waste products. They also serve to restrict the influx of antimicrobial agents and maintain low intracellular concentrations. In addition, alterations leading to decreased porin production or changes in the structure of porins that reduce their affinity for a drug can alter a resistance phenotype. In *E. coli*, major Omps include OmpA, OmpC, and OmpF. These proteins function as channels through which many small molecules, such as nutrients and antimicrobial agents, diffuse. Alteration or loss of OmpC and OmpF has been linked to decreased susceptibility to several antimicrobial agents, especially the carbapenems. Healthcare-associated pathogens showing a decrease or loss of porin synthesis are often observed in combination with other resistance mechanisms, resulting in MDR pathogens.

Efflux

Intrinsic resistance of bacteria to certain antimicrobials is caused by the activity of efflux systems and impermeability (Fig. 12.6). **Efflux pumps** are found in gram-positive and gram-negative bacteria and function as transporter proteins for the export of toxic substances and antimicrobial agents from the interior of the cell to the external environment. Efflux pumps are naturally occurring and are present in susceptible and resistant microorganisms. Efflux mechanisms can confer resistance to a particular antimicrobial agent, a class of agents, or several unrelated antimicrobials resulting in MDR.

Based on the amino acid sequence and energy source used to export their substrates, bacterial efflux transporters are classified into five major superfamilies: (1) the major facilitator superfamily (MFS), (2) the resistance nodulation cell division (RND) superfamily, (3) the small MDR family, (4) the ATP-binding cassette (ABC) superfamily, and (5) the

multidrug and toxic effects family. Of these, only the transporters in the ABC superfamily are primary transporters and hydrolyze ATP by ATPases to provide the energy required for active transport of antimicrobial agents and other toxic molecules. The rest of the families are secondary transporters that use a proton or sodium gradient as an energy source. The MFS dominates in gram-positive bacteria, whereas the RND superfamily is unique to gram-negative bacteria. The intrinsic efflux mechanism of resistance is chromosomally located and is activated by environmental signals or by mutation in regulatory genes.

In the RND superfamily, the *mexAB-oprM* operon in *P. aeruginosa* regulates porin and pump genes. This genetic locus plays a major role in the intrinsic resistance of pseudomonads and is a primary reason why infections caused by members of this genus are difficult to treat. This three-component efflux pump provides an exit portal for numerous agents, including quinolones, tetracyclines, macrolides, chloramphenicol, β -lactams, and meropenem, but not imipenem. The emerging resistance to imipenem in *P. aeruginosa* has been associated with the loss of the specific channel OproD, which facilitates the uptake of imipenem.

Bacterial cross-resistance to multiple antimicrobials can be mediated by efflux pumps capable of using multiple substrates. Exposure of a microorganism possessing an efflux pump to any one substrate belonging to a similar or different substrate profile used by that pump results in overexpression and consequent cross-resistance to all other substrates. Antimicrobials are no exception; in the *mexAB* system of *P. aeruginosa*, mutants that overproduce MexAB are less susceptible to fluoroquinolones, β -lactams, chloramphenicol, trimethoprim, and the antiseptic triclosan.

Efflux-mediated resistance to the oxazolidinones and streptogramins has been identified in gram-negative bacteria and *E. faecalis*, respectively. *E. faecalis* appears to be intrinsically resistant to dalbavancin, a streptogramin component of quinupristin-dalbavancin. The efflux mechanism designated *lsa* for *lincosamide and streptogramin A resistance* shows similarities to members of the superfamily of transporter proteins known as *ABC transporters* and influences resistance to quinupristin-dalbavancin and clindamycin. Most bacteria possess numerous efflux pumps; however, only a few per species appear to contribute resistance to antimicrobial agents used in clinical practice.

Enzymatic inactivation

Bacteria can produce enzymes that destroy the antimicrobial agents before they are able to reach their targets. Enzymatic inactivation of antimicrobial agents is one of the most intrinsic and acquired resistance mechanisms for β -lactam antibiotics, the class of agents most used to treat bacterial infections. They consist of four major groups: penicillins, cephalosporins, monobactams, and carbapenems (see Table 12.2) and differ from each other in their basic ring structure and moieties attached to the rings (see Fig. 12.4). All four groups have a four-member β -lactam ring susceptible to the hydrolytic activity of β -lactamases, the most widespread mechanism of bacterial resistance to this class of antibiotics (Fig. 12.7). β -Lactamases selectively hydrolyze the β -lactam ring to form the microbiologically inactive penicilloic acid. Currently, more than 1000 β -lactam resistance genes have been identified.

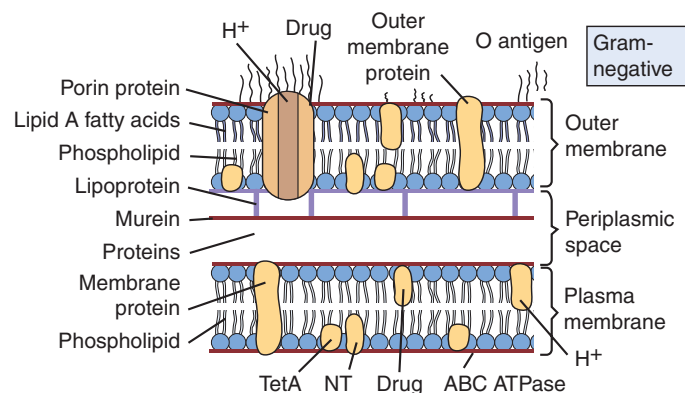


Fig. 12.6 Active efflux in gram-negative bacteria mediated by transmembrane proteins located in cytoplasmic and outer membranes. *ABC ATPase*, ATP-binding cassette family pumps; H^+ , efflux pumps driven by proton motive force mediated by the counterflow of protons; *NT*, nutrient transport; *TetA*, tetracycline-specific transporter pump.

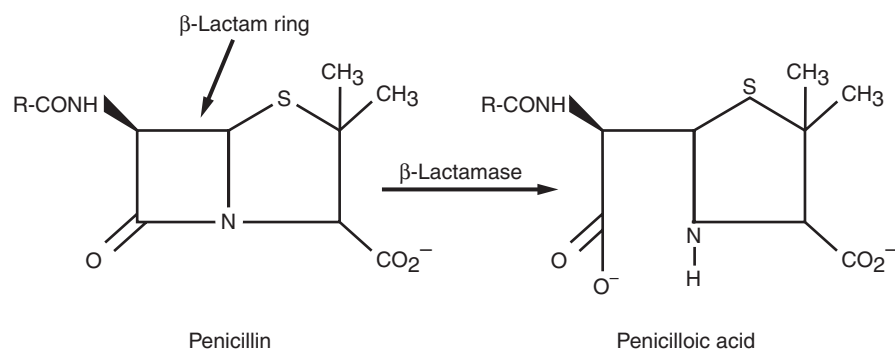


Fig. 12.7 β -Lactamase hydrolyzes the β -lactam ring portion of the penicillin molecule. The hydrolysis results in the formation of penicilloic acid, which does not have antibacterial activity.

β -Lactamases hydrolyze β -lactam antibiotics using two distinct mechanisms: a metallo-based mechanism and a serine-based mechanism. They are typically grouped into four classes, A to D, based on amino acid sequence similarity. Class A, C, and D enzymes use serine for β -lactam hydrolysis, whereas class B metalloenzymes require divalent zinc ions for substrate hydrolysis (see Table 12.3).

β -Lactam antibiotics act by binding to PBPs, bifunctional transglycosylases-transpeptidases responsible for the cross-linking (polymerization) of glycan strands (transglycosylation) and cross-linking of stem (backbone) peptide strands (transpeptidation). The similarities of class A, C, and D β -lactamases to mechanistic and architectural structures of PBPs suggest that the serine-based enzymes evolved from PBPs. Because of their structural similarity to PBPs, β -lactamases can serve as receptors for β -lactam antibiotics entering the cell. It is the binding equilibrium between PBP, β -lactamases, and the β -lactam antibiotic that determines the fate and ultimate survival of the microorganism. In addition, the observation that β -lactamases compete with PBPs for the antibiotic leads to the logical conclusion that the development of structural analogues of the β -lactam that bind the β -lactamase would enhance the activity of the β -lactam antibiotic. This observation led to the development of the **β -lactamase inhibitors (BLIs) clavulanic acid, sulbactam, and tazobactam**. The BLIs are structural analogues of the β -lactam antibiotics and function as substrates for β -lactamase, thus reducing their detrimental effects on the β -lactam antibiotic. When used jointly with β -lactam antibiotics, these inhibitors enhance the *in vitro* microbiological and clinical activity of the antibiotic.

Class A and C β -lactamases are considered the most clinically important, with class A enzymes primarily found on plasmids and constitutively expressed; class C enzymes are usually chromosomally located and inducible by exposure to β -lactams. In gram-negative bacteria, the β -lactamases are localized to the periplasmic space, where they act on incoming β -lactam antibiotics. In gram-positive bacteria, β -lactamases are secreted as exoenzymes and offer less protection to the microorganism. Almost all gram-negative bacteria mediate intrinsic (chromosomal) resistance by the enzymatic inactivation of penicillin class antibiotics exemplified by the class C β -lactamases. For example, *Citrobacter freundii*, *Klebsiella aerogenes*, and *P. aeruginosa* are clinically important healthcare-associated pathogens encoding chromosomal versions of class C β -lactamases.

Most class B metallo-dependent enzymes are chromosomally encoded cephalosporinases, and their expression can be constitutive or inducible. These β -lactamases are usually expressed in clinically important healthcare-associated pathogens such as *Stenotrophomonas maltophilia*, *Klebsiella pneumoniae*, and *P. aeruginosa*. Although the genes that regulate the production of these enzymes are commonly found on the chromosome, they can escape to plasmids and become transmissible, as has occurred for the class C enzyme encoded by *bla*CMY, which is widespread in enteric bacteria.

Acquired mechanisms of resistance

As noted earlier, the resistance mechanisms and genes that mediate resistance have presumably evolved in organisms that produce antimicrobial agents, so the agents are not effective against the producing organism. Furthermore, organisms that coevolved with antimicrobial-producing organisms may have acquired new functions in normal housekeeping genes to help detoxify antimicrobials. Thus by the process of natural selection, target organisms can acquire, evolve, and disseminate resistance determinants and use multiple combinations of intrinsic and acquired drug resistance strategies to survive toxic environments. This section focuses on acquired mechanisms such as efflux, modification of existing antimicrobial targets, acquisition of new targets, and production of enzymes that inactivate the antimicrobial.

Efflux

Although efflux plays a major role in intrinsic resistance, changes in the cell wall proteins can also result in novel acquired traits. In addition, some efflux pump genes have translocated to plasmids, which can be acquired by horizontal gene exchange. In general, multidrug efflux genes are broadly conserved in bacteria and are chromosome-encoded. On the other hand, drug-specific efflux genes are generally encoded by plasmids, so their acquisition is compounded by their association with MDR. An efflux pump encoded by the *mef* gene in *Streptococcus* is an example of an acquired macrolide resistance. The genetic element carrying *mefA* appears to be widely disseminated in *S. pneumoniae* and is transferable to other streptococcal species and other genera. *S. pneumoniae* isolates with efflux resistance are susceptible to clindamycin and usually have erythromycin minimum inhibitory concentrations (MICs) ranging from 1 to 16 $\mu\text{g/mL}$. Because the *mef*

phenotype demonstrates resistance to erythromycin but susceptibility to clindamycin, surveillance studies monitoring *S. pneumoniae* susceptibility patterns of erythromycin and clindamycin have revealed that the most prevalent mechanism of macrolide resistance in the United States is efflux mediated.

Currently, numerous plasmid-encoded acquired efflux genes that confer resistance to different antimicrobial agents have been detected in a variety of organisms. For example, plasmid-encoded tetracycline resistance efflux genes *Tet* (A), *Tet* (B), *Tet* (C), *Tet* (D), *Tet* (E), *Tet* (G), *Tet* (H), *Tet* (J), *Tet* (K), *Tet* (L), *Tet* (Y), *Tet* (Z), *Tet* (30), and *Tet* (39) have been detected in many gram-negative bacteria and attribute to most resistance phenotypes encountered in nature.

Target site modification

Modification of a target can reduce the binding affinity of the antimicrobial agent for the target. Modification of target sites occurs primarily by chromosomal mutation, as observed with the quinolone and oxazolidinone antimicrobials, and by enzymatic alteration of macrolide, glycopeptide, and β -lactam antibiotic target sites.

Chromosomal mutation

Quinolones target DNA gyrase and topoisomerase IV, inhibiting DNA synthesis. Although plasmid-mediated quinolone resistance genes (termed *qnr*) exist, the primary resistance mechanism to this antimicrobial class is caused by mutations in the genes encoding these DNA topoisomerases producing amino acid changes. Mutations are generally localized to the amino-terminal domains of GyrA and ParC, termed the *quinolone resistance-determining region* (QRDR). These mutations occur equally in the *gyrA* and *gyrB* subunits, but isolates from clinical settings usually demonstrate an exclusive prevalence for mutations in *gyrA*.

In *E. coli*, alterations in *gyrA* resulting in amino acid substitutions occur predominantly in the QRDR between positions 67 and 106. Although the presence of a single mutation in the QRDR of *gyrA* usually results in high-level resistance to nalidixic acid, the presence of additional mutations in *gyrA* and/or in another target such as *parC* is required to produce high levels of resistance to fluoroquinolones. Conversely, in gram-positive bacteria, first-step mutations leading to fluoroquinolone resistance occur in *parC* or *parE* subunits of topoisomerase IV, and second-step mutations occur in *gyrA*. Interestingly, first-step mutations in *gyrA* do not cause elevated MICs in *S. aureus*, suggesting that the primary target of the quinolone is topoisomerase IV.

β -Lactam antibiotics kill *S. pneumoniae* by targeting endogenous high-molecular-weight PBPs PBP1a, PBP1b, PBP2a, PBP2b, and PBP2x. Mutations in these PBPs lead to alteration of PBPs, which result in reduced affinity for β -lactam antibiotics. The levels of resistance are dependent on the number of mutations and PBPs involved. Mutations in PBP2 or PBP2x mediated by amino acid changes near the active site region of the PBP result in low-level resistance. Mutations in any of these transpeptidases-transglycosylases result in elevated MICs, and high-level resistance is the result of mutation in all five PBPs. Resistance to the oxazolidinones and streptogramin agents is also mediated by changes in the target site, leading to reduced affinity of the antimicrobial for its target.

Ribosomal mutation

Vancomycin-resistant *E. faecalis* mutants contain amino acid changes in domain V of the peptidyltransferase center of 23S rRNA. The most frequently encountered change is a transversion from guanine to uracil at position 2576, a mutation previously described in *E. faecium* isolated from patients who developed oxazolidinone resistance while receiving therapy. Resistance to the streptogramin antibiotic quinupristin-dalfopristin in *S. aureus* clinical isolates has been linked to alterations in ribosomal proteins with reduced affinity for streptogramin. The presence of the mutant proteins reduces the affinity of quinupristin for its target but does not affect the activity of dalfopristin; therefore the MIC susceptibility of pathogens containing this mutation increases, and there is a corresponding loss in the combined action of both antibiotics.

Enzymatic target site alteration

Enzymatic alterations of antimicrobial agent targets result in reduced affinity of the agents for their microbial targets and are exemplified by erythromycin ribosomal methylase (ERM) and by reprogramming of the peptidoglycan termini. Macrolides such as erythromycin bind the 50S subunit of the ribosome at the peptidyltransferase cavity in the proximity of the A and P loops and near adenine 2058 of 23S rRNA. Monomethylation or dimethylation of the amino group in the adenine residue of 23S rRNA results in reduced affinity of the macrolide for its target site and in elevated MICs.

Resistance to macrolides is mediated by an *ERM* gene found on plasmids and Tns that allow broad dissemination to many bacterial species. Currently, more than 20 classes of *erm* genes have been identified. Methylation of the 23S rRNA also results in cross-resistance to the lincosamide family and streptogramin B class of antibiotics. Cross-resistance to all three antibiotic groups is known as the *macrolide-lincosamide-streptogramin B (MLS) resistance phenotype*. Although MLS antibiotics are chemically distinct, they have a similar mode of action. Presumably, methylation leads to a conformational change in the ribosome that results in decreased affinity for all MLS antibiotics because the binding sites for these drugs overlap. Expression of the MLS resistance phenotype can be constitutive or inducible. However, the type of expression is not related to the classes of the *erm* gene; rather it depends on a regulatory region upstream of the methylase structure gene.

The glycopeptide vancomycin is used to treat enterococcal infections, in particular endocarditis, and is the drug of choice for the treatment of MRSA. Despite early attempts to avoid the overuse and subsequent emergence of resistance to vancomycin, widespread use resulted in the emergence of VRE isolates in health care settings. Currently, there are two choices approved for the treatment of VRE: quinupristin-dalfopristin (brand name Synercid) and linezolid (brand name Zyvox). Several agents have been approved for vancomycin-susceptible *Enterococcus* infections (e.g., daptomycin, tigecycline).

Proteins encoded by the *vanA* and *vanB* genes confer resistance to vancomycin, which is associated with alteration of the vancomycin-binding site in the cell wall and is clinically important in enterococci. *Enterococcus* spp. containing the VanA phenotype are highly resistant to vancomycin (MICs $\geq 64 \mu\text{g/mL}$) and teicoplanin (MICs $\geq 16 \mu\text{g/mL}$). The *vanA* gene resides on plasmids and on Tns that mediate spread of

the determinant. Conversely, the *vanB* determinant appears to be on large chromosomal elements. The VanB phenotype exhibits a range of susceptibilities and resistance to vancomycin but remains susceptible to teicoplanin. The VanA phenotype can be induced by vancomycin and teicoplanin, which is controlled by *vanS* and *vanR* genes, a regulatory pair that combine sensor and response regulators responsible for the control and expression of inducible vancomycin resistance.

The transmissibility of *vanA* is of great concern in the medical community, particularly transmission to MRSA, because vancomycin is the treatment of choice for MRSA. The emergence and genetic analysis of a high-level vancomycin-resistant *S. aureus* isolate (MIC = 1024 µg/mL) of clinical origin revealed the presence of a conjugative plasmid harboring Tn 1546 (*vanA*) that induces multiresistance. Potential spread of this strain in health care settings would have grave consequences for the continued therapeutic usefulness of vancomycin.

Acquisition of new targets

Microorganisms also become resistant by acquiring cellular targets with reduced affinity for the antimicrobial agent. MRSA emerged soon after the introduction of methicillin into clinical medicine in the 1960s and exemplifies the acquisition of a new target by a pathogen to respond to exposure to the toxic effects of the agent. *S. aureus* evaded the antimicrobial activity of methicillin by acquiring a mobile element carrying a staphylococcal cassette chromosome *mec*, which confers resistance to methicillin. The mobile DNA element encodes a triad of genes, *mecR1-mecI-mecA*. The *mecA* gene is responsible for methicillin resistance and encodes a new PBP, PBP2a (also PBP2a'), a bifunctional transglycosylase-transpeptidase with reduced affinity for β -lactam antibiotics, including penicillins, cephalosporins, carbapenems, and penems. The origin of the *mecA* gene remains unknown, as does the molecular basis of its nonsusceptibility to β -lactams.

Target site substitution operates in resistance to folate pathway inhibitors. Sulfonamides competitively compete for the dihydropteroate synthetase enzyme active site and block the formation of nucleotide precursors. Resistance in gram-negative and gram-positive bacteria is usually caused by the acquisition of a new enzyme that is unaffected by sulfonamides. The *sulI* and *sulII* genes, which encode drug-resistant dihydropteroate synthetases, have been found in many gram-negative bacteria. The *sulI* gene is almost always linked to other resistance genes and is located in conserved regions of integrons in Tn21-like elements carried by large, conjugated plasmids. The *sulII* gene is frequently linked genetically to a streptomycin-resistance gene on broad-host-range plasmids and on small nonconjugative plasmids.

Enzymatic inactivation of antimicrobial agents

The acquisition of enzymes that inactivate antimicrobial agents directly is one of the first mechanisms of resistance identified in bacteria and is a successful strategy used by many microorganisms to survive the action of several drug classes. Examples are enzymes that mediate hydrolysis of the β -lactam ring of β -lactam antibiotics and modification of structural moieties of aminoglycoside antibiotics. The activity of the β -lactamases was discussed previously as an intrinsic mechanism of resistance.

As mentioned earlier, β -lactamases are classified into four major classes: the serine enzymes A, C, and D and the metalloenzymes B. Class A and C β -lactamases are regarded as the most clinically significant. Class A enzyme genes are primarily found on plasmids and are constitutively expressed, whereas class C enzyme genes are primarily found on chromosomes and are inducible upon exposure to β -lactams.

The most important clinical class A β -lactamases are the families TEM, SHV, CTX, PSE, and KPC. They are usually found in gram-negative bacteria. TEM-1 is typically found in *E. coli*, *K. pneumoniae*, *K. aerogenes*, and *Haemophilus influenzae* and is generally located on transmissible plasmids. SHV-1 is usually found in clinical isolates of *K. pneumoniae* and appears to be the most common of the chromosomal β -lactamases found in this species.

With the emergence of plasmid-mediated β -lactamase resistance, concern regarding the possible widespread transmission of these resistance mechanisms led to the development of new oxyimino- β -lactam antibiotics resistant to the hydrolysis of class A β -lactamases. Although these parenterally administered cephalosporins and monobactams are excellent therapies, their continued use provided selection pressure for variants of existing β -lactamases that could hydrolyze these new antibiotics. These extended-spectrum β -lactamases (ESBLs) are known to be derivatives of the common TEM, SHV, and OXA type B β -lactamases that differ by one or more amino acid substitutions near the reactive site of the enzyme. The modified amino acid sequence yields a protein capable of extending substrate use and increasing affinity for the antibiotic target molecule. Interestingly, the enhanced spectrum of the ESBLs also makes them better substrates for BLIs. ESBLs can also hydrolyze specific sets of penicillins, cephalosporins, and monobactams, although not all ESBLs can hydrolyze all cephalosporins equally well.

CTX-M enzymes emerge from plasmid-mediated ESBLs following their mobilization from the chromosome of *Kluyvera* spp. They also belong to the class A β -lactamases and have greater activity against cefotaxime and ceftriaxone, but generally not against ceftazidime, which has important implications for laboratory detection. CTX-M enzymes show only approximately 40% identity with TEM and SHV, two commonly isolated β -lactamases. More than 125 CTX-M variants are currently known and are further divided into five subfamilies based on their amino acid homology—CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25. CTX-Ms have mainly been found in strains of Enterobacterales. The successful spread of CTX-M genes may be influenced by several factors, including the clonal spread of strains carrying the resistance genes, the mobile genetic element responsible for its capture, and the plasmids on which it is found. CTX-M enzymes are the predominant ESBL type in parts of South America and are widespread in Europe, with CTX-M-14, CTX-M-3, and CTX-M-2 being the most common. CTX-M-15 is currently the most widespread type in *E. coli* worldwide, including in the United States.

Metallo- β -lactam resistance is increasingly being reported and is of global health interest because many metallo- β -lactams are capable of crossing species; they are the most encountered transferable carbapenemases. The most common types of metallo- β -lactams found in Enterobacterales are the VIM and IMP types and, more recently, the newest subgroup of metallo- β -lactams, named *New Delhi metallo- β -lactamase-1*

(NDM-1). The enzyme NDM-1 was first discovered in 2008 in a Swedish patient who had traveled to New Delhi and acquired a urinary tract infection caused by a carbapenem-resistant *K. pneumoniae* strain that was resistant to all antimicrobials tested, except colistin. It is now widespread and reported from many countries in Europe, Asia, Africa, Australia, and North America. Molecular examination of the first NDM-1 gene revealed that NDM-1 hydrolyzes penicillins, cephalosporins, and carbapenems, but not the monobactam, aztreonam.

Another β -lactamase reported with increasing frequency worldwide is “oxacillinase-48” (OxA-48), which belongs to class D of the Ambler classification. This enzyme was named *oxacillinase* because it was first recognized to inactivate oxacillin and methicillin. It is now known to also inhibit the cephalosporins, extended-spectrum cephalosporins, and carbapenems. This can render agents that are often the last defense against MDR bacteria ineffective.

Cephalomycins (CMYs), AmpC-like β -lactamases that hydrolyze cephalosporins and cephamycins such as cefoxitin and ceftriaxone, have recently been detected in Enterobacteriales. CMYs have been reported in *K. pneumoniae*, *E. coli*, *Proteus mirabilis*, *Salmonella*, and *K. aerogenes* from many countries, including the United States. *Salmonella* resistance to ceftriaxone is a significant public health concern because ceftriaxone is a drug of choice to treat *Salmonella* infection in humans, particularly young children. CMY is a class C β -lactamase encoded by *bla*CMY genes. To date, more than 90 *bla*CMY variants have been described, and they can reside on plasmids or chromosomes. Many studies have shown that *bla*CMY genes are transferable by conjugation and transformation.

In addition to degradation, enzyme-mediated resistance mechanisms can exert their effects by modification of the drug. As noted, amino and hydroxy radicals of aminoglycoside antibiotics form hydrogen bonds with the 30S ribosomal subunit, thus preventing mRNA translation. Resistance to aminoglycosides is mediated by efflux, changes in target site, impermeability, or enzymatic modification of the amino and hydroxy moieties appended to the cyclitol rings. The most clinically relevant is enzymatic modification of the drug.

There are three classes of aminoglycoside-modifying enzymes: aminoglycoside *N*-acetyltransferase (AAC), aminoglycoside *O*-adenyltransferase (ANT), and aminoglycoside *O*-phosphotransferase (APH). Inactivation of the aminoglycoside by the aminoglycoside-modifying enzymes is a result of the transfer of a functional group to the aminoglycoside; AAC transfers the acetyl group from acetyl-CoA to the NH_2 group, ANT transfers the nucleotide triphosphate, and APH transfers the phosphoryl group from ATP to the OH or NH_2 group. In general, only phosphorylating enzymes confer very high levels of resistance. These resistance genes are typically found on plasmids in gram-negative bacteria including the Enterobacteriales, *P. aeruginosa*, and *Acinetobacter* spp.

Another example of enzymatic alteration of a synthetic antimicrobial involves a plasmid-borne variant of an aminoglycoside acetyltransferase, AAC (6')-Ib, which acetylates fluoroquinolones and reduces their activity. Because fluoroquinolones are completely synthetic, naturally evolving antimicrobial resistance genes have not been considered a threat in the reduction of their activity. This variant enzyme acts

against some fluoroquinolones and affects resistance to aminoglycosides, implying that plasmids bearing this gene may coselect for multiple resistance.

β -Lactamase inhibitors

To enhance the therapeutic range of β -lactam antibiotics that are inactivated by β -lactamases, chemicals called BLIs were developed. They act by preventing the degradation of β -lactam antibiotics, thus extending the range of bacteria the β -lactam antibiotics are active against. Use of BLIs is important in treating infections caused by gram-negative bacteria, as β -lactamase production is an important contributor to β -lactam resistance in these pathogens. Whereas the gram-positive pathogen *S. aureus* produces β -lactamases, the resistance of this species to antibiotics is caused mostly by variant PBP_s.

BLIs have little antimicrobial activity of their own; thus they are used in combination with specific antibiotics. The majority of the currently used BLIs have a core structure resembling a β -lactam antibiotic. However, recently two BLIs whose core structure is not like a β -lactam antibiotic (non- β -lactam BLIs) have been developed: **avibactam** and **relebactam**. Currently available BLIs are effective against Ambler class A β -lactamases (tazobactam, clavulanate, and sulbactam), C β -lactamases, and D β -lactamases (avibactam). Because they have a similar chemical structure, the BLIs are processed in a way like a β -lactam antibiotic to form an initial covalent intermediate. Unlike the case with the β -lactam antibiotic, however, the formed covalent bond intermediate is very stable. This persistence between the BLI and the β -lactamase binding site deactivates the β -lactamase, preventing it from inactivating other β -lactam molecules. The antibiotics and BLIs are coformulated (Table 12.4) with similar half-lives; this is done for dosing convenience and to minimize the development of resistance.

Ambler class B β -lactamases cleave β -lactams by a mechanism like that of metalloproteases. As no covalent intermediate is formed, the mechanism of action of available BLIs is not applicable. Thus the spread of bacterial species expressing metallo- β -lactamases, such as the New Delhi metallo- β -lactamase, has caused considerable concern.

Table 12.4 β -Lactamase inhibitors

β -Lactamase inhibitors with β -lactam core	Coformulated antibiotic
Clavulanic acid or clavulanate	Amoxicillin (Augmentin), ticarcillin (Timentin)
Sulbactam	Ampicillin (Unasyn), cefoperazone (Sulperazon)
Tazobactam	Piperacillin (Zosyn and Tazocin), ceftolozane (Zerbaxa)
β -Lactamase inhibitors with non- β -lactam core	
Avibactam	Ceftazidime (Avycaz)
Relebactam	Imipenem-cilastatin (Recarbrio)
Vaborbactam	Meropenem (Vabomere)

Dissemination of resistance determinants

Case check 12.3

Mobile elements such as plasmids, Tns, and integrons are not essential for bacterial proliferation and survival. However, they can carry genes that impart selective advantages to the host bacterium, such as antimicrobial resistance genes that allow microorganisms to survive exposure to antimicrobial agents. Such mobile elements can move from one part of a genome to another or between genomes via lateral gene transfer (LGT). LGT is an important strategy for bacteria to spread antimicrobial resistance. Thus resistance genes in any organism at any location in the microbial biosphere can be mobilized and spread globally. The bacterium causing the infection in the Case in Point had the opportunity to acquire drug-resistant genes via mobile elements found in the environment.

Resistance is an evolutionary strategy that allows microorganisms to survive chemically hostile environments, such as those posed by exposure to antimicrobial agents. However, the evolution and vertical transfer of these resistance determinants to progeny is only part of the survival strategy used by microorganisms. Bacteria have the remarkable ability to share genetic resources, including resistance genes via LGT. LGT is the most important mechanism for bacteria to spread antimicrobial resistance, and it occurs on a global scale. Thus resistance genes in any organism anywhere in the microbial biosphere can be mobilized and spread globally. LGT requires at least two independent processes: (1) physical movement of DNA and (2) incorporation of DNA into the receiving genome. The mechanisms of transfer of resistance determinants from one bacterium to other bacteria are **conjugation**, **transformation**, and **transduction** (see [Chapter 1](#)). The mobile elements that carry the resistance genes transferred through LGT are plasmids, Tns, and integrons.

Plasmids are circular DNA elements that replicate independently of the chromosome and can acquire and exchange information with the chromosome and other host-resident plasmids. Plasmids can be conjugative and self-transmissible or nonconjugative, requiring mobilization by conjugative plasmids. Plasmid-encoded traits are typically not essential for bacteria to survive but carry genes that impart some selective advantage to the host bacterium, such as virulence determinants, adhesions, and antimicrobial drug resistance. Plasmids that carry resistance genes are called *R plasmids* or *R factors* and play a key role in spreading antimicrobial resistance genes. Since their discovery in the 1950s, antimicrobial resistance plasmids have been increasingly associated with gram-positive and gram-negative bacterial pathogens and with commensal organisms.

Plasmid-associated resistance genes have been characterized for most clinically available antimicrobials. It is not uncommon for a single plasmid to mediate resistance to multiple antimicrobials and be shared among different bacterial genera simultaneously. For example, the IncA/C MDR plasmid carries resistance to many different classes of antimicrobials, including β -lactams, aminoglycosides and aminocyclitols,

tetracyclines, phenicols, quaternary ammonium compounds, sulfonamides, and trimethoprim. This plasmid has been broadly disseminated among MDR zoonotic pathogens, such as *Yersinia pestis*, *Yersinia ruckeri*, *E. coli*, and various serovars of *Salmonella*.

Until recently, quinolone resistance was believed to arise solely from chromosomal mutations in genes encoding target enzymes or via active efflux. It is now clear that plasmid-mediated quinolone resistance occurs and is conferred by unusual resistance determinants, including *qnr*, *aac(6')-Ib-cr*, and *qepA* (an efflux pump belonging to the major facilitator subfamily). The *qnr* determinant was discovered in 1998 in a *K. pneumoniae* isolate from Birmingham, Alabama, and was found in an integron-like structure on the MDR plasmid pMG252. The plasmid encoded Qnr proteins derived from *E. coli*, *Klebsiella oxytoca*, and *K. pneumoniae* isolates recovered from different geographic sources (China, Europe, and the United States) show almost identical amino acid residues, indicating that these proteins likely have a common origin. It appears that the *qnr* genes were acquired from chromosomal genes in aquatic bacteria and now are usually associated with mobilizing or transposable elements on plasmids. There is also evidence supporting a role for these plasmids in linking resistance to quinolones and ESBLs. This is especially troubling, given the importance of fluoroquinolones and β -lactams in clinical medicine.

Even more troubling in relation to plasmid-encoded antimicrobial resistance is the emergence of the plasmid-mediated, mobilized colistin resistance gene (*mcr-1*) in Enterobacterales. The *mcr-1* gene confers resistance to many of the last-resort antimicrobials, including colistin. This is extremely concerning from a public health perspective because *mcr-1* is transferred via horizontal gene transfer in Enterobacterales. The *mcr-1* gene is distributed on at least five continents, and isolates with the *mcr-1* gene have been recovered from human infections, including in the United States.

Tns are DNA elements that encode transposition and excision functions and can move from one place on the chromosome and/or plasmid to another. Transposase, an enzyme that facilitates nonhomologous recombination, mediates the transposition event. Much like plasmids, Tns are also capable of carrying antimicrobial resistance genes and shuttle these determinants among plasmids and the chromosome. Tns can be very simple, composed of the **insertion sequence (IS)** elements, or much more complicated, as in composite Tns. ISs are short DNA sequences that act as a simple transposable element. ISs have two major characteristics: (1) they are small relative to other transposable elements, and (2) they code only for proteins used in the transposition activity. These proteins usually include transposase, which catalyzes the enzymatic reaction allowing the IS to move, and also one regulatory protein, which stimulates or inhibits the transposition activity.

ISs can form composite transposable genetic elements, such as Tn10, with the IS elements forming the proximal and distal ends and genetic material that codes for antimicrobial resistance located in between. ISs associated with antimicrobial resistance include IS256, which was linked to amikacin, gentamicin, and oxacillin resistance in *Staphylococcus pseudintermedius*. Several resistance determinants in many different bacterial species are transmitted via composite Tns. Transmission of a Tn from one bacterial species to another can

be accomplished by insertion into a conjugative plasmid or via a conjugative Tn. Conjugative Tns appear to be a hybrid between Tns and plasmids and have been identified in many gram-negative and gram-positive bacteria.

Plasmid NR1 isolated from *Shigella flexneri* is the archetype plasmid responsible for the dissemination of antimicrobial resistance utilizing these elements. NR1 is a 94.5-kb multiple antimicrobial resistance plasmid that carries the genes for self-transmissibility and autonomous replication. NR1 also carries a resistance-determining region bound by direct repeats and is self-transmissible as Tn2670. Nested within Tn2670 is Tn21, a transposon of particular interest not only because it exhibits the transpositional characteristic of Tns and the presence of resistance determinants, but also because it contains a potentially mobile DNA element, the integron.

Integrans are genetic elements that capture mobile gene cassettes by site-specific recombination. Integrans share a common recombination system composed of a gene (*intI*) that encodes a site-specific recombinase (IntI) and an adjacent primary recombination site (*attI*) into which gene cassettes can be integrated. The integron also contains a promoter that directs transcription of any cassette inserted into the 5' conserved sequence of the integron. The *attI* is a 59-base element recognized by IntI that mediates insertion into the integron. Multiple gene cassettes can be arranged in tandem, and more than 60 distinct cassettes have been identified. Cassette-associated genes have been shown to confer resistance to β -lactams, aminoglycosides, phenicols, trimethoprim, streptothricin, and quaternary ammonium compounds.

So far, five classes of integrans have been described based on the *intI-attI* combinations. Among enteric bacteria and several different genera, class I integrans are the most frequently identified. Integrans are usually identified in conjunction with streptomycin and trimethoprim-SMZ-resistant bacteria isolated from food animals and human infections. The inclination to exchange genes increases concern about the possible spread of drug resistance determinants from commensal or nonclinical organisms in animals and humans to human pathogens. Integrans can be chromosomally encoded and are not independently mobile; however, they are usually mobilized on plasmids and Tns. Clonal expansion of drug-resistant bacteria together with the movement of antimicrobial resistance gene cassettes within and between integrans play important roles in the spread of MDR bacteria.

As noted previously, vancomycin is an important antibiotic used to treat infections caused by MRSA. However, a MRSA isolate of clinical origin was found to contain a conjugative MDR plasmid that encoded resistance to vancomycin, trimethoprim, β -lactams, aminoglycosides, and hospital disinfectants. Genetic analysis revealed the presence of a multiresistant conjugative plasmid harboring Tn1546 (VanA). A Tn1546-like transposable genetic element encoding for transposition, regulation of VanA expression, and resistance is also found in enterococci. The data suggest that the interspecies transfer of Tn1546 occurs from a coresident isolate of *E. faecalis* to MRSA. Resistance to vancomycin can be spread by the transposition of 1547 to a conjugative plasmid and transferred by conjugation to recipient strains or by excision and circularization of the Tns, followed by conjugation.

In addition to self-transmissible mobile DNA elements, antimicrobial resistance determinants can be spread among

bacteria via uptake of naked DNA from the surrounding environment (transformation) or by infection with a bacteriophage carrying resistance genes (transduction). Transformation was the first mechanism of DNA transfer to be discovered among prokaryotes and involves DNA scavenging by a bacterium after the death and deterioration of a nearby bacterium. The DNA in a dead bacterium degrades and is broken into fragments that are released into the surrounding milieu, which can then be internalized by transformation-competent recipients. If antimicrobial resistance genes are included in the degraded DNA, they can be expressed when the DNA is taken up by a nearby bacterium and incorporated into the bacterial genome.

Genetic exchange via transduction involves bacteriophage infection of a bacterium, phage replication, packaging of some of the bacterial DNA with the phage DNA (which may include resistance determinants) in the phage capsid, with lysis of that bacterium, and bacteriophage infection of subsequent bacteria. On subsequent infection, those resistance determinants can be transferred to the newly infected bacterium. The contribution of transformation and transduction in the evolution of MDR is difficult to assess, but laboratory demonstrations indicate that it might play a role in antimicrobial resistance development.

Nanotechnology to deliver therapeutic agents

Nanoparticles are less than 100 nm in size and have unique biological, physical, and chemical properties at these dimensions. The nanomaterial can be loaded with drugs, biomolecular diagnostic tools, and contrast agents that can help locate the agents and understand their action in real time. These tools can help us understand the mechanisms of drug resistance more accurately. Furthermore, based on this information, new formulations can be developed to overcome MDR. In many cases, the unique properties of nanomaterial facilitate the fast diffusion of the drug to the infection, which can significantly enhance treatment.

Nanomedicine is being tested to deliver various therapeutic drugs to tissue. This technology has the potential to treat infectious diseases and cancer. For example, mRNA vaccines for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) utilize lipid nanoparticles to deliver the mRNA encoding for the SARS-CoV-2 spike protein into the cell and have demonstrated high efficacy after two doses. Nanoencapsulation delivery systems are used to increase the circulation time of drugs, which allows the drugs to reach their destination in the body without degradation. Nanoencapsulation can increase the solubility and improve the pharmacokinetic profiles of insoluble drugs, and in many cases, targeted drug delivery greatly enhances the bioavailability to the target tissues. Encapsulating the drug allows more surface modification, and encapsulated ingredients can be stabilized for longer periods.

Several nanoparticle-based drug delivery systems are approved for clinical use to treat a variety of infectious diseases. Bacterial infections are known to trigger inflammatory reactions that can stimulate vascular permeability. Increased permeability and dysfunctional lymphatic drainage have

been shown to facilitate the accumulation of drug-loaded nanoparticles at the infection site. Under physiologic conditions, pathogenic bacteria have a negative surface charge. Cationic nanoparticles are capable of binding to bacteria by electrostatic interactions. Nanoparticles also have been coated with ligands that bind specifically to bacterial receptors. To reach intracellular bacteria, nanoparticles have been coated with ligands binding to macrophage receptors. The macrophage takes up the nanoparticle, delivering the loaded drug.

As mentioned previously, combination therapy with synergistic drugs is becoming more commonly used to combat MDR bacteria. A difficulty with this strategy is the differing pharmacokinetics, biodistribution, and membrane-penetrating properties of different drugs. Loading multiple drugs in nanoparticles can overcome these difficulties. For example, isoniazid and rifampin have been loaded into liposomes. This therapeutic strategy has been shown to be more effective in treating tuberculosis compared with free drugs at the same dosage.

Nanoparticle delivery systems include liposomes, polymeric nanoparticles, and dendrimers. Liposomes are closed, continuous bilayered structures of synthesized polymers that can be used for nanoencapsulation. They are stable and perfect for storing and stabilizing the drug delivery system. In the liposome artificial membrane, small, closed vesicles can be formed that make up a lipid bilayer. This lipid bilayer encloses a small droplet of water, approximately 10 to 20 nm. These small structures can be used to deliver the drug.

Nanoshells are about 5 to 10 times smaller than liposomes, with a pore size of around 2 nm. Nanoshells consist of vesicular porous structures that allow them to be effective in targeting the drug. They are synthesized from silica or calcium phosphate. Nanoshells are biodegradable and trap the drugs in the interior space, actively targeting the drug site. They release drugs from their internal cavities as the structure erodes. The toxicity levels for nanoshells are lower than for other delivery systems but are subject to further investigation.

Another way of delivering drugs is by using cochleates. These are small, stable lipid structures composed of a bilayer sheet that is rolled up into a spiral-like structure. The benefits of this structure are that cochleates have higher stability, and they can deliver drugs to negatively and positively charged species. They are used typically in oral delivery, as opposed to other methods.

POINTS TO REMEMBER

- Resistance to antimicrobial agents may be genetically intrinsic or acquired; both types can be found in the same pathogen.
- β -Lactam antibiotics, which work by inhibiting cell wall synthesis, contain a four-member ring and include the penicillins, cephalosporins, monobactams, and carbapenems.
- Quinolones interfere with DNA replication by targeting DNA gyrases and topoisomerases.
- Protein inhibitors include aminoglycosides, tetracyclines, macrolides, streptogramins, chloramphenicol, and linezolid.
- BLIs are used with β -lactam antibiotics to increase the activity of the antibiotic.

- Impermeability and efflux mechanism of resistance can produce multidrug-resistant phenotypes.
- Conjugation, transformation, and transduction are the mechanisms of transfer of resistance determinants among bacteria.
- The mobile elements that carry resistance genes during lateral gene transfer are plasmids, Tns, and integrons.
- A single organism can acquire multiple mechanisms of resistance, including different mechanisms of conferring resistance to the same compound.
- Antimicrobial mechanisms of resistance and their dissemination are not static processes.
- Nanoparticles are being used to deliver various drugs, including antimicrobial agents and vaccines, into tissue.

LEARNING ASSESSMENT QUESTIONS

1. Antibiotics can be:
 - a. Natural molecules.
 - b. Synthetic molecules.
 - c. Semi-synthetic molecules.
 - d. All of the above.
2. Which of the following antimicrobials act on cell wall biosynthesis?
 - a. Macrolides and phenicols
 - b. Tetracycline and streptomycin
 - c. β -Lactams and glycopeptides
 - d. Fluoroquinolone and sulfamethoxazole
3. Which of the following antimicrobials are new analogues of older classes?
 - a. Glycylcycline from tetracyclines
 - b. Fluoroquinolone from glycopeptides
 - c. Ketolides from macrolides
 - d. Both a and c
4. The most used inhibitors of cell wall biosynthesis act on:
 - a. Synthesis of precursors in the cytoplasm.
 - b. Transpeptidation linking and maturation.
 - c. Insertion of glycan units into the cell wall.
 - d. Both b and c
5. Which of the following antimicrobial classes does not include semi-synthetic and natural derivatives?
 - a. Aminoglycosides
 - b. Fluoroquinolones
 - c. Glycopeptides
 - d. β -Lactams
6. Intrinsic drug resistance is:
 - a. Often caused by genetic change.
 - b. Found in only a few members (strains) within a species.
 - c. Transmitted vertically.
 - d. Typically found on mobile elements.
7. Which mechanisms of antimicrobial resistance are intrinsic and acquired?
 - a. Efflux
 - b. Biofilm
 - c. Acquisition of new targets
 - d. Gram-negative outer membrane
8. Plasmids can contain which of the following?
 - a. Transposons
 - b. Insertion sequences
 - c. Integron cassettes
 - d. All of the above

9. Transposons do not contain which of the following?
 - a. Transposase gene
 - b. Cytoplasmic membranes
 - c. Excision protein genes
 - d. Antibiotic resistance determinants
10. Which of the following characteristics apply to efflux pumps?
 - a. They are found in gram-positive and gram-negative bacteria.
 - b. They are transporter proteins.
 - c. They have single or multiple substrates.
 - d. All of the above.
11. Enzymatic modification of aminoglycosides is *not* caused by which of the following?
 - a. Dimethylation
 - b. *N*-acetylation
 - c. *O*-phosphorylation
 - d. *O*-adenylation
12. Which of the following is *not* a characteristic of integrons?
 - a. Potentially mobile element
 - b. Gene cassettes
 - c. Peptidoglycan
 - d. 59-bp element
13. Which of the following antimicrobials acts predominantly on cell membrane integrity?
 - a. Aminoglycosides
 - b. Polymyxins
 - c. Tetracyclines
 - d. Penicillins
14. Which of the following is a non- β -lactam β -lactamase inhibitor?
 - a. Relebactam
 - b. Ticarcillin
 - c. Clavulanate
 - d. Sulbactam
15. Serine-based β -lactamases appear to have evolved from the penicillin-binding proteins of bacteria. True or false?

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Antimicrobial susceptibility testing

Paula C. Mister and Donald C. Lehman

CHAPTER OUTLINE

Procedures in antimicrobial susceptibility testing, 272

Reasons and indications for performing antimicrobial susceptibility tests, 273

Factors to consider when determining whether susceptibility testing is warranted, 273

Selecting antimicrobial agents for testing and reporting, 274

Selection of test batteries, 274

Reporting of susceptibility test results, 274

Traditional antimicrobial susceptibility test methods, 275

Inoculum preparation and use of McFarland standards, 275

Dilution susceptibility test methods, 276

Disk diffusion testing, 279

Interpretation of in vitro antimicrobial susceptibility test results, 282

Modified test methods for slow-growing or fastidious bacteria, 284

Additional organism and antimicrobial agent testing concerns, 287

β -Lactamase tests, 291

Etest, 292

Automated antimicrobial susceptibility test methods, 293

Principles of technologies used, 293

Automated systems, 293

Quality control of antimicrobial susceptibility tests, 295

Selecting an antimicrobial susceptibility test method, 298

Rapid susceptibility determination, 299

Special antimicrobial susceptibility tests, 299

Minimum bactericidal concentration test, 300

Controlling test variables, 301

Interpretation concerns, 301

Time-kill assays, 301

Synergy tests, 302

Serum bactericidal test, 302

Measurement of antimicrobial agents in serum and body fluids, 302

Biological assays, 302

Immunoassays, 303

Chromatographic assays, 303

Bibliography, 305

OBJECTIVES

After reading and studying this chapter, you should be able to:

1. Explain the rationale behind the performance of antimicrobial susceptibility tests.
2. Describe the method for selection of specific drugs in testing and reporting.
3. Define minimal inhibitory concentration (MIC).
4. Justify the methods used for determining MICs.
5. Explain how zone-interpretive criteria used with the disk diffusion test are established.
6. List the variables that must be controlled when antimicrobial susceptibility tests are performed.
7. Justify test modifications for antimicrobial susceptibility testing of *Helicobacter/Campylobacter* spp., *Streptococcus* spp. (including *Streptococcus pneumoniae*), *Haemophilus* spp., *Neisseria gonorrhoeae*, *Neisseria meningitidis*, and obligate anaerobes.
8. Explain the principles behind automated antimicrobial susceptibility test methods.
9. Discuss several commercially available antimicrobial susceptibility test systems in current use.
10. Justify testing bacterial isolates for β -lactamase.
11. List the organisms for which β -lactamase testing is indicated.
12. Explain the reliable methods for detection of methicillin-resistant *Staphylococcus aureus*, vancomycin-intermediate *S. aureus*, and vancomycin-resistant *S. aureus*.
13. Discuss use of the D-zone test.
14. Describe the significance of high-level aminoglycoside resistance in enterococci.
15. Describe extended-spectrum β -lactamases and carbapenemases and assays to detect them.
16. Justify testing for extended-spectrum β -lactamases and carbapenemases.
17. Discuss quality control procedures for antimicrobial susceptibility tests.
18. Describe how antibiograms can be used to help verify the accuracy of results generated by testing patient isolates.
19. Discuss situations in which cumulative antibiograms may help guide antimicrobial therapy.

20. Explain how to select a particular susceptibility test method for routine use.
21. Explain the antimicrobial susceptibility test interpretive results of *nonsusceptible*, *susceptible*, *intermediate*, and *resistant*.
22. Explain the function of the International Organization for Standardization.
23. Describe the minimum bactericidal concentration (MBC) test and the indications for performing this test.
24. Justify determining the MBC of bacterial isolates.
25. Define *synergism*, *antagonism*, and *indifference* as related to testing combinations of antimicrobial agents.
26. Describe the serum bactericidal test and the indications for performing this test.
27. Explain how molecular probes might be used to detect antimicrobial resistance.
28. Discuss methods used for measuring concentrations of antimicrobial agents in serum and body fluids and when such tests are used.

KEY TERMS

Antagonism	Minimal inhibitory concentration (MIC)
Antibiogram	Minimum bactericidal concentration (MBC)
β -Lactamases	Nonsusceptible
Borderline oxacillin-resistant isolates	Oxacillin-salt screen plate
Breakpoint	Penicillinase-producing <i>Neisseria gonorrhoeae</i> (PPNG)
Carbapenemase-producing Enterobacterales (CPE)	Penicillinase-resistant penicillins
Chromosomally mediated resistant <i>Neisseria gonorrhoeae</i> (CMRNG)	Persister cells
Clinical and Laboratory Standards Institute (CLSI)	Resistant
Cumulative antibiogram	Selective reporting
D-zone test	Serum bactericidal test (SBT)
Etest	Susceptible
Extended-spectrum β -lactamase (ESBL)	Synergism
Heteroresistant	Time-kill assay
High-level aminoglycoside resistance	Tolerance
Indifference	Trailing
Intermediate	U.S. Food and Drug Administration (FDA)
Kirby-Bauer test	Vancomycin agar screen plate
McFarland turbidity standards	Vancomycin-intermediate <i>Staphylococcus aureus</i> (VISA)
<i>mecA</i> gene	Vancomycin-resistant enterococci (VRE)
Methicillin-resistant <i>Staphylococcus aureus</i> (MRSA)	Vancomycin-resistant <i>Staphylococcus aureus</i> (VRSA)
	Zone of inhibition

Case in point

The microbiology laboratory supervisor was reviewing patient reports that the floating laboratory scientist had generated earlier in the day. She saw a susceptibility report for *Staphylococcus aureus* isolated from a patient's wound that included the following disk diffusion testing results: cefazolin-S, ceftiofur-R, clindamycin-R, erythromycin-R, penicillin-R, and vancomycin-S. However, there was no result for oxacillin. The supervisor realized that the laboratory

scientist who had reported these results might have forgotten the current method for detecting **methicillin-resistant *Staphylococcus aureus* (MRSA)** and reporting rules for β -lactam agents and MRSA. She reminded the laboratory scientist of the special testing method and reporting rules, and the laboratory scientist corrected and rereleased the report, which was as follows: clindamycin-R, erythromycin-R, oxacillin-R, penicillin-R, and vancomycin-S.

Issues to consider

After reading the patient's case history, consider:

- Use of surrogate antimicrobial agents to test for resistance or susceptibility for the drug reported
- Editing certain susceptible results to resistant, and not reporting related drugs, when bacteria with specific types of resistance are encountered
- Modifications of standard tests that might be necessary to detect specific types of emerging resistance

Procedures in antimicrobial susceptibility testing

Antimicrobial susceptibility testing is performed on bacteria and fungi isolated from clinical specimens to determine which antimicrobial agents might be effective in treating infections caused by these organisms. Only organisms that are likely to be contributing to an infection should be tested. Testing of organisms that are not involved in an infection is misleading to the physician and could lead to unnecessary treatment and a more serious infection with development of antimicrobial resistance.

A major goal of the clinical microbiology laboratory is to identify organisms that are causing infections. Often, these organisms must be distinguished from the normal microbiota that can reside at the site of the infection, although in some situations the microbiota that resides at the site of the infection may be contributing to the infection. Therefore it must be determined which organisms from a specimen are clinically relevant and will be identified and tested for susceptibility to antimicrobials. Most microbiology laboratories have guidelines for determining when and on which microorganism susceptibility testing will be done. When in doubt about the significance of an organism, it is best to discuss the situation with the attending physician and microbiology laboratory director.

In clinical laboratories, susceptibility testing on bacteria can be performed by classic disk diffusion or dilution (**minimal inhibitory concentration [MIC]**) methods. More commonly, one of several different types of commercial automated antimicrobial susceptibility test systems may be used. Protocols that describe these methods are published and frequently updated by the **Clinical and Laboratory Standards Institute (CLSI)** in the United States. The European counterpart is The European Committee on Antimicrobial Susceptibility Testing (EUCAST). Worldwide, antimicrobial susceptibility testing is guided by the International Organization for Standardization (ISO). This organization is a worldwide federation of national standards bodies. The work of preparing international standards is conducted

through ISO technical committees, whose main task is to prepare international standards. Publication as an international standard requires approval by at least 75% of the member bodies casting a vote. In all cases, it is important to maintain an awareness of which antimicrobial agents are appropriate to test, the reliability of various test systems for detecting antimicrobial resistance, and strategies for effectively communicating results on laboratory reports to those who must be informed.

Reasons and indications for performing antimicrobial susceptibility tests

Antimicrobial susceptibility testing should be performed on a bacterial isolate from a clinical specimen if the isolate is determined to be a probable cause of the patient's infection and the susceptibility of the isolate to antimicrobials cannot be reliably predicted based on previous experience with the bacteria at a specific health care facility. Although information can be determined on the susceptibility of specific bacteria from the literature, there may be significant differences at a specific health care facility, based on the patient population. Susceptibility tests are not performed on bacteria that have a predictable susceptibility pattern to antimicrobial agents. For example, group A β -hemolytic *Streptococcus* is not routinely tested because it is universally susceptible to penicillin, the drug of choice in treating infections caused by this bacterium. In contrast, the recommended agent for treating *Staphylococcus aureus* infections is oxacillin, but not all *S. aureus* isolates are susceptible to oxacillin. Thus susceptibility testing is routinely indicated for an *S. aureus* isolate that is suspected of causing an infection.

Susceptibility testing of isolates can also provide information on decreases in the susceptibility of bacteria to antimicrobials. A separate set of critical MICs other than the MICs used to judge a bacterium susceptible or not susceptible to an antimicrobial are developed for this purpose. These values are termed *epidemiologic cutoff values* (ECVs). ECV MICs separate bacterial populations into those likely to be wild type (susceptible) versus those that are becoming resistant, either by mutation or acquisition of resistance genes. When the ECV MIC value is achieved, it is an indication that the bacterium is likely developing resistance to the antimicrobial. This information can be used to adjust the use of that antimicrobial in the health care facility to delay or avoid those species from becoming resistant to the antimicrobial. Such adjustments include discontinuing the use of the drug for a time, reserving use of the drug for specific patients, using the drug in combination with another, and generally using an antimicrobial of a different class to treat infections caused by that bacterium.

Factors to consider when determining whether susceptibility testing is warranted

In addition to the unpredictable susceptibility of a potential pathogen, other important factors must be considered when

determining whether antimicrobial susceptibility testing is warranted, including:

- Body site from which the bacterium was isolated
- Presence of other organisms and quality of the specimen from which the organism was grown
- Host's status

Body site

Susceptibility tests are not routinely performed on organisms isolated from an anatomic site for which they are normal inhabitants. For example, *Escherichia coli* is normal microbiota in the lower gastrointestinal tract and therefore would not be tested when isolated from stool. However, *E. coli* from a blood culture would be tested because blood is normally sterile. Similarly, viridans group streptococci represent normal microbiota in throat specimens and would not be routinely tested. Coagulase-negative staphylococci isolated from multiple blood cultures would be tested; but because coagulase-negative staphylococci are commonly found on skin surfaces, these bacteria would not normally be tested when isolated from superficial wound specimens. Yeasts isolated in low numbers in vaginal specimens or in the throat, if other microbiota is present, would not be considered significant. Testing of normal microbiota isolates, or isolates likely to represent contamination or colonization, should be avoided because reporting antimicrobial susceptibility results may encourage a physician to treat a nonpathogen and prevent further investigation of the true cause of the patient's problem.

Presence of other bacteria and quality of the specimen

The isolation of an organism in pure culture is less likely to represent contamination than a mixed culture. The presence of more than two species at greater than 10^5 colony-forming units (CFU) per milliliter isolated from urine suggests contamination, and these organisms may not require susceptibility testing; however, a pure culture of *E. coli* at more than 10^5 CFU/mL would likely represent true infection and would be tested. A few colonies of *Klebsiella pneumoniae* in the presence of oropharyngeal microbiota in a sputum culture may not be significant. However, in the absence of oropharyngeal microbiota, a few colonies of this species, particularly if gram-negative bacilli were noted on a direct Gram stain of the sputum, can be significant and warrant susceptibility testing.

Host status

The host status of the patient often influences susceptibility testing decisions. In an immunocompromised patient, species usually viewed as normal microbiota might be responsible for an infection and therefore may require susceptibility testing. Also, in patients who are allergic to penicillin and have a β -hemolytic streptococcal infection (e.g., group A or group B), erythromycin or clindamycin are alternative drugs of choice. Occasionally β -hemolytic streptococci are resistant to these agents, so testing might be performed.

Selecting antimicrobial agents for testing and reporting

Currently, more than 50 antimicrobial agents are in use for treating bacterial and fungal infections. Although hundreds more exist, many of these have comparable clinical efficacy. Each laboratory must determine which agents are appropriate for routine testing against various organisms in its setting. Testing and reporting protocols are formulated with input from drug prescribers, representatives from the microbiology laboratory, infectious diseases and other services, the institution's pharmacy, and the therapeutics committee regarding the clinical usefulness of various agents at an institution. Antimicrobial package inserts written by the **U.S. Food and Drug Administration (FDA)** should be consulted for information concerning the dosing and indications for which the antimicrobial was approved and the performance of the antimicrobial agent during initial clinical trials.

It is important that the antimicrobial drugs used by the laboratory match the institutional formulary as closely as possible. From the laboratory perspective, the limiting factor for the number of drugs tested is usually the number that can be practically tested with a specific method. For example, the standard disk diffusion test for bacteria uses a 150-mm agar plate, which can accommodate no more than 12 disks. In the case of MIC test panels, 12 to 15 antimicrobials can be tested on one panel. Some automated antimicrobial susceptibility systems can test more drugs at one time.

The patient population must be considered in the choice of antimicrobial agents to be tested. Some agents are contraindicated in pediatric patients (e.g., fluoroquinolones, which can impair cartilage development, and tetracycline, which damages developing teeth). Emphasis should be placed on testing oral agents when dealing with outpatient specimens. Additional guidelines for developing a testing and reporting strategy are found in the CLSI documents for disk diffusion or MIC testing. These documents include tables that list primary and secondary agents appropriate for testing against various organism groups. CLSI guidelines are updated at least annually.

Selection of test batteries

Generally, a laboratory will define a battery of 10 to 15 antimicrobial agents for routine testing against the Enterobacterales, *Pseudomonas* spp., nonfastidious gram-negative bacilli (e.g., *Acinetobacter* spp., *Stenotrophomonas maltophilia*, and *Burkholderia cepacia*), staphylococci, and enterococci. Sometimes a separate battery is performed for urine isolates, representing drugs appropriate for treating urinary tract infections (UTIs). A supplemental battery that contains antimicrobial agents with enhanced activity may be included by laboratories that encounter a significant number of bacteria resistant to the more commonly used antimicrobials.

Case check 13.1

Specific rules are developed by laboratories for dealing with specific susceptibility test results based on institutional data and data from outside sources. These include reporting antimicrobial results on appropriate bacteria (e.g., gram-positive vs. gram-negative) from relevant clinical sites.

Reporting of susceptibility test results

Because the identity of the bacterial isolate is sometimes unknown when the susceptibility test is performed, some drugs that are inappropriate to report may be tested on an isolate. In such cases, a drug should not be indiscriminately reported because results can be misleading. Some drugs may appear active against certain species in vitro but are inappropriate for clinical use (e.g., most cephalosporins against methicillin-resistant *Staphylococcus aureus* [MRSA], trimethoprim-sulfamethoxazole against enterococci). The final decision about the antimicrobials to report is made once the identity of the isolate is known (sometimes a preliminary identification is sufficient), along with the overall susceptibility results and specimen source.

Reporting protocols should be developed following discussion with infectious disease clinicians, pharmacists, and others who have clinical experience with the antimicrobial therapy practices of the institution. A primary tenet of antimicrobial therapy is to use the least toxic, most cost-effective, and most clinically effective agents and to refrain from use of costly, broader-spectrum agents. Achieving physician compliance with this objective can be difficult. If all drugs tested are reported, inappropriate antimicrobial prescribing may occur, so most laboratories refrain from reporting broad-spectrum agents if narrower-spectrum agents are active in vitro. CLSI provides guidance for development of a selective or so called *cascade* reporting protocol. In addition, antimicrobial stewardship programs in many institutions help educate and guide clinicians in appropriate and responsible antimicrobial therapy.

For several organism groups, the CLSI categorizes antimicrobial agents into four groups. An example of a listing for Enterobacterales is seen in [Box 13.1](#). As a general guideline, it is suggested that within an antimicrobial class, primary (group A) agents be reported first and secondary (group B) agents be reported only if one of the following conditions exists:

- The isolate is resistant to the primary agents.
- The patient cannot tolerate the primary agents.
- The infection has not responded to the primary agents.
- A secondary agent would be a better clinical choice for the infection (e.g., meningitis).
- Bacteria were isolated from another site, and a secondary agent might be useful for treating both infections.

For example, a primary cephalosporin, such as cefazolin (a first-generation cephalosporin), would be a reasonable choice for a susceptible *E. coli*, and secondary cephalosporins, such as cefuroxime (second-generation cephalosporin) or cefotaxime (third-generation cephalosporin), would generally not be required. An exception would occur with meningitis because third-generation cephalosporins cross the blood-brain barrier much more effectively than their first-generation counterparts. Gentamicin is usually the aminoglycoside of choice for treating serious infections caused by gentamicin-susceptible *Pseudomonas aeruginosa*, and tobramycin or amikacin may be considered for gentamicin-resistant isolates. Aminoglycosides are not effective for treating meningitis because they do not readily cross the blood-brain barrier.

A secondary agent may also be reported if the patient has a polymicrobial infection, and a secondary (but not a primary)

BOX 13.1 Suggested groupings of antimicrobial agents: Enterobacterales**Group A primary test and report**

Ampicillin^a
Cefazolin^b
Gentamicin^a
Tobramycin^a

Group B primary test report selectively

Amikacin^a
Amoxicillin–clavulanic acid
Ampicillin–sulbactam
Azithromycin^c
Ceftazidime–avibactam
Ceftolozame–tazobactam
Imipenem–relebactam
Meropenem–vaborbactam
Piperacillin–tazobactam
Cefuroxime
Cefepime
Cefotetan
Cefoxitin
Cefotaxime^{a,b} or ceftriaxone^{a,b}

Cefiderocol
Ciprofloxacin^a
Levofloxacin^a
Doripenem
Ertapenem
Imipenem
Meropenem
Trimethoprim–sulfamethoxazole^a

Group C supplemental report selectively

Aztreonam
Ceftazidime
Ceftaroline
Chloramphenicol^{a,d}
Tetracycline^e
Group U supplemental for urine only
Cefazolin
Fosfomycin^f
Nitrofurantoin
Sulfisoxazole
Trimethoprim

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^aWhen fecal isolates of *Salmonella* and *Shigella* spp. are tested, only ampicillin, a fluoroquinolone, and trimethoprim–sulfamethoxazole should be tested and reported routinely. In addition, a third-generation cephalosporin should be tested and reported (also chloramphenicol and azithromycin if requested) for extraintestinal isolates of *Salmonella* spp. Routine testing of nontyphoidal intestinal *Salmonella* is not indicated, but testing on all *Shigella* isolates is indicated. For *Salmonella* and *Shigella* isolates, aminoglycosides, first- and second-generation cephalosporins, and cephamycins can appear active in vitro but should not be reported as they are ineffective clinically.

^bCefotaxime/ceftriaxone should be reported on CSF isolates instead of cefazolin.

^cReport for *S. Typhi* or *Shigella* spp. only.

^dNot routinely reported on isolates from urine.

^eOrganisms susceptible to tetracycline are considered susceptible to doxycycline and minocycline; organisms intermediate or resistant to tetracycline may be susceptible to doxycycline, minocycline, or both.

^fTest and report on urinary isolates for *E. coli* and *E. faecalis* only.

agent would more likely be effective against all pathogens present. Similarly, a secondary agent may be reported if the patient has a disseminated infection, and a secondary agent would more likely be effective at all sites. Agents with very broad-spectrum activity or increased potency (group C) may be tested and reported for the reasons listed for secondary agents. In addition, group C agents would be considered for routine testing if a particular institution encounters large numbers of isolates resistant to group A and group B agents. Finally, agents with activity only in the urinary tract should be reported only on isolates from urine; these drugs concentrate in the urine and are clinically ineffective in treating other infections.

Traditional antimicrobial susceptibility test methods

Inoculum preparation and use of McFarland standards

Inoculum preparation

Inoculum preparation is one of the most critical steps in susceptibility testing. Inocula are prepared by adding cells from four to five isolated colonies of similar colony morphology growing on a noninhibitory agar medium to a broth medium

and then allowing them to grow to log phase at 35° C for 2 to 6 hours, until they reach the McFarland 0.5 standard (see later). Four to five colonies, rather than a single colony, are selected to minimize the possibility of testing a colony that might have been derived from a susceptible mutant. Inocula can also be prepared by suspending colonies grown overnight on an agar plate directly in broth or saline. This direct inoculum suspension preparation technique is required for staphylococci and for bacteria that grow unpredictably in broth (e.g., fastidious bacteria) but can be used for any organism. Because it does not rely on growth in an inoculum broth, the use of fresh (16- to 24-hour) colonies is imperative.

McFarland turbidity standard

The inoculum concentration of bacteria to be tested must be standardized. False-susceptible results may occur if too few bacteria are tested, and false-resistant results may be the outcome of testing too many bacteria. The most widely used method of inoculum standardization involves comparing the turbidity of the inoculum preparation with **McFarland turbidity standards**. McFarland standards can be prepared by adding specific volumes of 1% sulfuric acid and 1.175% barium chloride to obtain a barium sulfate solution with a specific optical density. The most used is the McFarland 0.5 standard, which contains 99.5 mL of 1% sulfuric acid and 0.5 mL of 1.175% barium chloride. This solution is dispensed into tubes comparable with those used for inoculum preparation, which are sealed tightly and stored in the dark at room

temperature. The McFarland 0.5 standard provides turbidity comparable with that of a bacterial suspension containing approximately 1.5×10^8 CFU/mL. Suspensions of latex particles can be used as a simpler, more stable alternative to barium sulfate to achieve turbidity comparable with that of the McFarland standard.

Inoculum standardization

To standardize the inoculum, the inoculated broth or direct suspension is vortexed thoroughly. The traditional method of comparison is to position the tube side by side with the McFarland 0.5 standard against a white card containing several horizontal black lines (Fig. 13.1) under adequate lighting. The turbidities are compared by looking at the black lines through the suspension. The suspension is too dense if it is more difficult to see the lines through the inoculum suspension than through the McFarland 0.5 standard. In such a case, the inoculum would be diluted with additional sterile broth or saline. If the test suspension is too light, more organisms are added, or the suspension is reincubated (depending on the inoculum preparation protocol) until the turbidity reaches that of the McFarland standard. Once standardized, the inoculum suspensions should be used within 15 minutes of preparation.

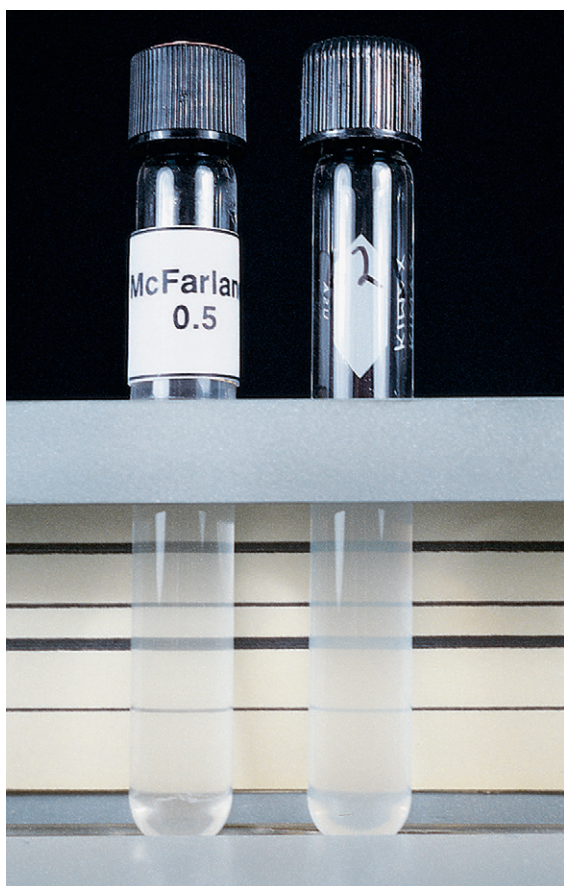


Fig. 13.1 *Left*, The tube contains a McFarland 0.5 turbidity standard. *Right*, The tube contains a test bacterial suspension that has turbidity greater than that of the McFarland standard; it is more difficult to see the black lines through the test suspension than through the McFarland standard. Sterile saline or broth must be added to the test suspension to dilute it until the turbidity matches that of the McFarland standard.

A convenient and more precise alternative to the traditional, highly subjective visual adjustment to match the McFarland standard is the use of a nephelometer or spectrophotometer. Several simple, commercially available benchtop instruments are commonly used for more objective standardization of bacterial inocula (Fig. 13.2).

Dilution susceptibility test methods

Principle

Dilution antimicrobial susceptibility test methods are used to determine the MIC, or the lowest concentration of antimicrobial agent required to inhibit the growth of the bacterium. Various concentrations of an antimicrobial agent are added to broth or agar media. Generally, serial twofold-dilution concentrations are tested (expressed in micrograms per milliliter). Concentrations chosen are those that are attainable in vivo following standard dosing of the antimicrobial agent. Because the concentration attainable in vivo differs with different agents, the ranges of concentrations tested also differ. For example, sustained concentrations greater than $8\mu\text{g/mL}$ for gentamicin and tobramycin cannot be safely attained in the patient; therefore the concentrations tested are generally in the range of 0.25 to $8.0\mu\text{g/mL}$. In contrast, much higher levels of the extended-spectrum penicillin, piperacillin, are attainable in vivo, and a range of 4 to $128\mu\text{g/mL}$ might be tested.

Once the MIC has been determined, the organism is interpreted as **nonsusceptible**, **susceptible**, **intermediate**, or **resistant** to each agent with the use of a table provided in the CLSI dilution testing document or in the FDA-approved package insert for the antimicrobial. *Susceptible* indicates that the bacteria are inhibited by achievable concentrations of the agent in vivo when the recommended dose is administered to



Fig. 13.2 Benchtop nephelometric-type devices can be used to standardize the turbidity of a test inoculum suspension to match that of a McFarland 0.5 turbidity (or other) standard.

treat an infection. *Intermediate* implies that antimicrobial MICs approach the usual blood and tissue levels, and response rates might be lower than for susceptible isolates. This applies to agents with lower toxicity that can be prescribed at higher-than-normal doses. *Resistant* implies that isolates are not inhibited by the usually achievable concentrations of the agent with normal dosage. *Nonsusceptible* is used for isolates for which only a susceptible breakpoint is designated because of the absence or rare occurrence of resistant strains. Both the FDA and the CLSI are involved in determining antimicrobial interpretive criteria for MIC test results. An example is shown in Table 13.1. Note that for some antimicrobial agents, different MIC interpretive criteria exist for different organisms or organism groups. For each antimicrobial agent, the MIC *breakpoint* separates susceptible from resistant results. Organisms with MICs at or below the breakpoint are susceptible, and those with MICs above that breakpoint are intermediate or resistant. Broth macrodilution, broth microdilution, and agar dilution methods are used for determining MIC values. These methods are briefly summarized in the following sections.

Antimicrobial stock solutions

Antimicrobial stock solutions used in MIC tests must be prepared from reference standard antimicrobial powders,

Table 13.1 Minimal inhibitory concentration interpretive standards for several organism groups ($\mu\text{g/mL}$)

Antimicrobial agent	Susceptible	Intermediate	Resistant
Ampicillin			
When testing Enterobacterales	≤ 8	16	≥ 32
When testing enterococci	≤ 8	—	≥ 16
When testing <i>Haemophilus</i> spp.	≤ 1	2	≥ 4
When testing viridans group streptococci	≤ 0.25	0.5–4.0	≥ 8
Gentamicin			
When testing Enterobacterales	≤ 4	8	≥ 16
When testing <i>Pseudomonas aeruginosa</i>	≤ 4	8	≥ 16
When testing staphylococci	≤ 4	8	≥ 16
Oxacillin			
When testing <i>Staphylococcus aureus</i>	≤ 2	—	≥ 4
When testing coagulase-negative staphylococci	≤ 0.5	—	≥ 1

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not from the pharmaceutical preparations administered to patients. Details of preparation are found in the CLSI protocols. Stock drug solutions must be stored frozen in non-frost-free freezers. Temperature at or below -60°C is optimal and necessary for more temperature-labile drugs such as imipenem and clavulanic acid; however, -20°C storage is acceptable for some agents. Antimicrobial solutions are not to be refrozen after thawing.

Broth macrodilution (tube dilution) tests

Broth dilution MIC tests performed in test tubes are referred to as broth macrodilution MIC or tube dilution MIC tests. Generally, a twofold serial dilution series, each containing 1 to 2 mL of antimicrobial agent, is prepared. Mueller-Hinton broth is the medium recommended for broth dilution MIC tests of nonfastidious bacteria. A standardized suspension of test bacteria is added to each dilution to obtain a final bacterial concentration of 5×10^5 CFU/mL. A growth control tube (broth plus inoculum) and an uninoculated control tube (broth only) as a sterility check are used with each assay. After overnight incubation at 35°C , the MIC is determined visually as the lowest concentration that inhibits growth, as demonstrated by the absence of turbidity.

Broth macrodilution is impractical for use as a routine method when several antimicrobial agents must be tested on an isolate or if several isolates must be tested. Some laboratories use broth macrodilution when it is necessary to test drugs not included in their routine test system or for fastidious bacteria that require special growth media. Also, this method can be used when **minimum bactericidal concentration (MBC)** end points are to be determined subsequently. The MBC test is discussed later in this chapter.

Broth microdilution tests

The broth macrodilution test has been miniaturized and adapted to multiwell microdilution trays (Fig. 13.3) for broth microdilution MIC testing. Plastic trays contain between 80 and 100 (usually 96) wells. Wells are filled with small volumes (usually 0.1 mL) of twofold dilution concentrations of antimicrobial agent in broth. Because of the large number of

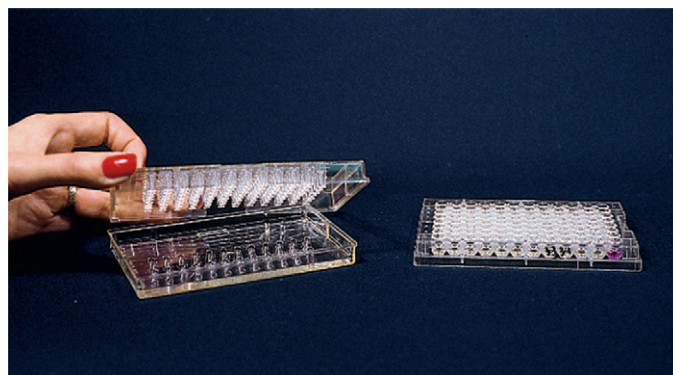


Fig. 13.3 Broth microdilution minimal inhibitory concentration (MIC) tray shown with an inoculum reservoir trough. A diluted inoculum suspension is placed in the reservoir trough. Then the prongs are dipped into the suspension, raised, and subsequently lowered into the wells of the broth microdilution MIC tray to inoculate all wells simultaneously.

wells, several dilutions of as many as 12 to 15 antimicrobial agents can be contained in a single tray that subsequently will be inoculated with one bacterial isolate. The inoculum suspension is prepared and standardized, as described earlier. An intermediate dilution of this inoculum suspension is prepared in water or saline, and a multipronged inoculator or other type of inoculating device is used to inoculate the wells to obtain a final concentration of approximately 5×10^5 CFU/mL (5×10^4 CFU/0.1-mL well). The actual dilution factor used for preparation of the intermediate dilution depends on the volume of inoculum delivered to each well by the inoculating device and the organism being tested. An example of this calculation is illustrated in Table 13.2. A growth control well and uninoculated control well are included on each tray. After overnight incubation at 35° C, the tray is placed on a tray-reading device to facilitate visual examination of each well (Fig. 13.4). Provided growth is adequate in the growth control well, the MIC for a particular drug is the lowest concentration showing no obvious growth.

Table 13.2 Sample calculations of dilution schema for preparation of inocula for broth microdilution minimal inhibitory concentration tests^a

Step	Resulting organism concentration
1. Standardize suspension to McFarland 0.5 standard	1.5×10^8 CFU/mL
2. Add 0.75 mL from step 1 to 25 mL water diluent (1:33 dilution)	$(4-5) \times 10^6$ CFU/mL
3. Use inoculator prong set to inoculate wells of MIC tray (each prong delivers 0.01 mL, which results in an additional 1:100 dilution)	$(4-5) \times 10^4$ CFU/100-μL well $(4-5) \times 10^5$ CFU/mL

^aCalculations shown here are based on use of inoculator prong set (each prong delivers 0.01 mL). Dilutions in step 2 differ, depending on the number of organisms in the initial suspension and the volume delivered to each well by the inoculating device.
CFU, Colony-forming units; MIC, minimal inhibitory concentration.



Fig. 13.4 Tray-reading stand with magnifying mirror (left) and light box (right). Following incubation, either of these devices is used to examine growth in the broth microdilution minimal inhibitory concentration trays. A tray is placed on the tray-reading stand, and wells are examined by looking into the magnifying mirror. A tray is placed over the hole in the light box, and light is allowed to shine through the tray to facilitate close examination of the wells.

Growth may be seen as turbidity, a haze, or a pellet in the bottom of the well. Results for quality control (QC) organisms should be read before reading results for patient isolates to determine whether the test was performed correctly.

Some laboratories use dispensing devices to prepare broth microdilution panels, but most laboratories purchase commercially prepared panels, frozen or freeze-dried. Commercially prepared panels must be stored and prepared for use as indicated by the manufacturer. Generally, frozen panels are thawed at room temperature just before inoculation. For dried panels, the dried or lyophilized drugs in the wells are reconstituted when the panels are inoculated. In addition to the panels, all manufacturers sell other materials needed for testing (e.g., inoculum broths, inoculum diluents, panel inoculators, reading devices). Usually, a variety of panels containing different drugs are available for testing various organism groups (e.g., gram-positive, gram-negative), and some panels are designed to include wells containing various biochemical reagents so organism identification and antimicrobial susceptibility testing can be done simultaneously in a single panel. Some companies have automated or semi-automated devices to facilitate inoculation and reading. These are discussed in the following sections.

Breakpoint (cutoff) minimal inhibitory concentration testing

A variation of the standard broth microdilution MIC panel is the inclusion of breakpoint testing, in which only one or a few concentrations of certain antimicrobial agents are tested on a single panel, in addition to standard MIC testing on other drugs. **Breakpoint (cutoff)** is the term applied to the concentration of an antimicrobial agent that coincides with a susceptible or intermediate MIC breakpoint for a particular drug. When two concentrations are tested and no growth is present in either well, the isolate is susceptible. When there is growth in the low concentration but no growth in the high concentration, the isolate has intermediate susceptibility, and a resistant isolate grows in both wells. The qualitative interpretation—nonsusceptible, susceptible, intermediate, or resistant—rather than an MIC value is reported. The primary advantage of breakpoint over full MIC testing for a drug is that a greater number of drugs can be tested on a single panel because a smaller number of wells is needed. The primary disadvantage of breakpoint testing is that a precise MIC is not obtained. Standalone breakpoint panels are generally not currently used clinically.

Trailing growth and skipped wells

Dilution methods sometimes produce an MIC end point that is not clear-cut, and growth in the wells may demonstrate trailing or skipped wells. **Trailing** involves heavy growth at lower concentrations followed by one or more wells that show greatly reduced growth in the form of a small button or a light haze. This commonly occurs with sulfonamides, trimethoprim, and trimethoprim-sulfamethoxazole; the mode of action of the agents allows the bacterial cells to grow through several generations before inhibition. In this case the trailing is ignored, and the end point is read as an 80% reduction in growth compared with the growth control. Trailing with most other drugs may represent contamination and should not be ignored unless it is known that trailing commonly occurs with the antimicrobial agent–organism combination.

Skipped wells involve growth at higher concentrations and no growth at one or more of the lower concentrations. This may occur because of contamination, improperly inoculated wells, improper concentrations of antimicrobial agent in the wells, the presence of unusual resistance with the test isolate (e.g., a small resistant subpopulation), or a combination of two or more of these factors. As with trailing, each skipped well occurrence must be evaluated individually to determine whether results are reportable. If any doubt exists about the validity of the results, they should not be reported, and the test should be repeated.

One shortcoming of the broth microdilution MIC method is its inability to produce a penicillin MIC that is consistently within the resistant range for staphylococci that are low-level, β -lactamase producers. If the penicillin MIC is $0.03\mu\text{g/mL}$ or lower, the isolate may be reported as penicillin-susceptible; a result of $0.25\mu\text{g/mL}$ or higher is considered resistant. It is recommended that an induced β -lactamase test be performed on isolates with penicillin MICs of 0.06 to $0.12\mu\text{g/mL}$. If the β -lactamase test result is positive, the isolate is reported as penicillin-resistant; if the result is negative, the isolate is reported as penicillin-susceptible.

Agar dilution tests

The MIC can also be determined using an agar dilution method. Specific volumes of antimicrobial solutions are dispensed into premeasured volumes of molten agar, which is subsequently poured into standard Petri dishes and then cooled. Mueller-Hinton agar is recommended for testing aerobic isolates; however, this can be supplemented with sheep's blood, to a final concentration of 5% sheep blood, or other nutrients for testing fastidious bacteria. A series of plates containing various concentrations of each antimicrobial agent and growth control plates without antimicrobial agent are prepared. A standard number of test bacteria (10^4 CFU for aerobes) are spot-inoculated onto each plate using a multi-pronged replicating device (Fig. 13.5). As many as 32 different

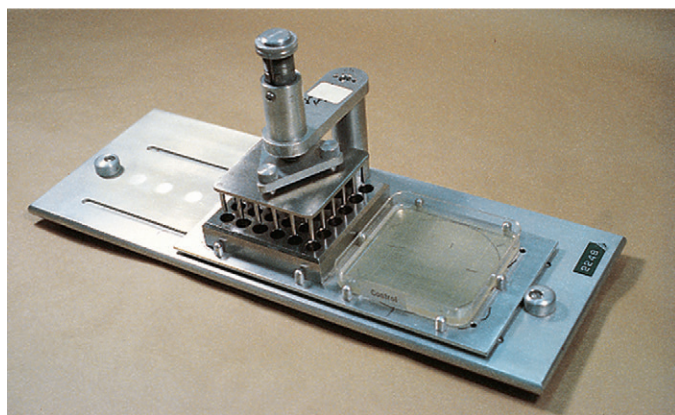


Fig. 13.5 Steer's replicator. The inoculator prongs are positioned above a 36-well seed trough that contains 36 different standardized inoculum suspensions. The handle on top of the prong unit is pressed to lower the prongs into all suspensions simultaneously, and when the prongs are raised, each contains a standardized volume of inoculum. The agar plate containing a defined concentration of antimicrobial agent is positioned under the prongs (the steel plate holding the agar plate slides back and forth), and the prongs are carefully lowered to the agar, at which point the inocula are deposited on the agar surface. This process is repeated until all antimicrobial-containing and control agar plates (without antimicrobial agent) have been inoculated.

isolates can be simultaneously inoculated onto each 100-mm round Petri dish; 100-mm square plates generally accommodate 36 isolates. After overnight incubation, the MIC is read as the lowest concentration of antimicrobial agent that inhibits the visible growth of the test bacterium (one or two colonies are ignored).

The shelf life of agar dilution plates is only 1 week for most antimicrobial agents stored at 2° to 8°C because many drugs are labile in this temperature range. Because plate preparation is laborious and this procedure is practical only if large numbers of isolates are tested, agar dilution is generally performed in research settings, although it is currently considered the primary method for antimicrobial susceptibility testing of obligate anaerobes and is the only accepted reference method for *Neisseria gonorrhoeae* and *Helicobacter pylori*.

Disk diffusion testing

Principle

The disk diffusion test, commonly known as the **Kirby-Bauer test**, has been widely used in clinical laboratories since 1966, when the first standardized method was described. Briefly, a McFarland 0.5 standardized suspension of bacteria (as described in Inoculum Preparation earlier) in Mueller-Hinton broth is swabbed over the surface of a standardized Mueller-Hinton agar plate, and paper disks containing specific concentrations of antimicrobial agent are placed onto the inoculated surface. After incubation of 16 to 18 hours, the diameters of the zones of inhibition around each disk are measured. The result is interpreted as nonsusceptible, susceptible, intermediate, or resistant to a particular drug according to preset criteria. This method has been standardized for common, rapidly growing bacteria; it should not be used for slowly growing bacteria and obligate anaerobes. An isolate of *Klebsiella* (*Enterobacter*) *aerogenes* tested by disk diffusion is shown in Fig. 13.6.

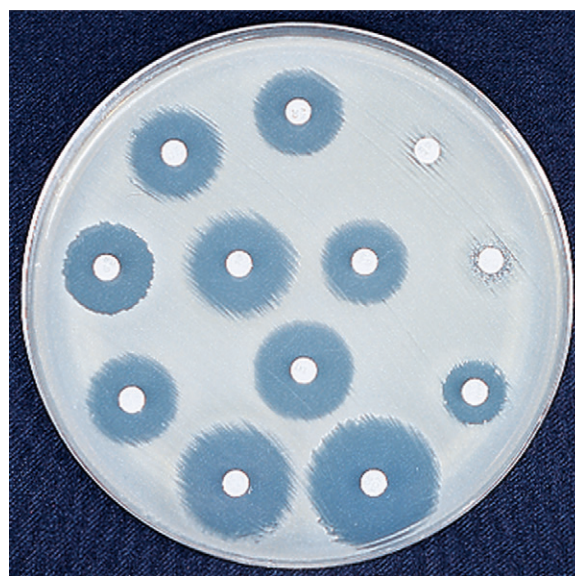


Fig. 13.6 *Klebsiella* (*Enterobacter*) *aerogenes* tested by the disk diffusion method. Zone measurements confirm that the isolate is susceptible to all agents tested, except ampicillin (at the 1-o'clock position) and cefazolin (at the 2-o'clock position). No zones are present for either of these agents.

Establishing zone diameter interpretive breakpoints

The disk diffusion test depends on the formation of an antimicrobial concentrations gradient as the antimicrobial agent radially diffuses into the agar. The drug concentration decreases at increasing distances from the disk. At a critical point, the amount of drug at a specific location in the medium is unable to inhibit the growth of the test organism, and a **zone of inhibition** is formed.

The zones of inhibition are related to MICs, and this relationship is used to determine the breakpoints for interpreting a particular zone measurement indicating that the isolate is nonsusceptible, susceptible, intermediate, or resistant. To establish breakpoints for a single agent, the first step is to determine the optimum concentration of drug to incorporate into the disk, which considers the drug's pharmacokinetic properties and some of its biochemical properties (e.g., molecular size, solubility, diffusibility in agar). Next, a sample of 150 to 200 isolates with comparable growth rates and differing susceptibility to the agent are tested by the disk diffusion test and a dilution MIC test, and results are plotted on a graph. For each isolate, the observed MIC is expressed in logarithmic form ($\log_2$) and plotted on the *y*-axis, and the corresponding zone measurement is plotted on the *x*-axis on an arithmetic scale (scattergram; Fig. 13.7). Zone diameter breakpoints are then selected based on a comparison of the susceptible, intermediate, and resistant MIC breakpoints with the zone diameters, while considering the typical in vivo achievable concentrations from standard dosing. The best zone diameter breakpoints are chosen to categorize isolates correctly with minimal interpretive errors.

With some of the newer, extremely potent drugs, resistant isolates may not presently exist, so only a susceptible criterion is defined; no intermediate- or resistant-zone interpretive criteria are specified. Further tests, possibly in a reference laboratory, should be performed on any isolate that is interpreted

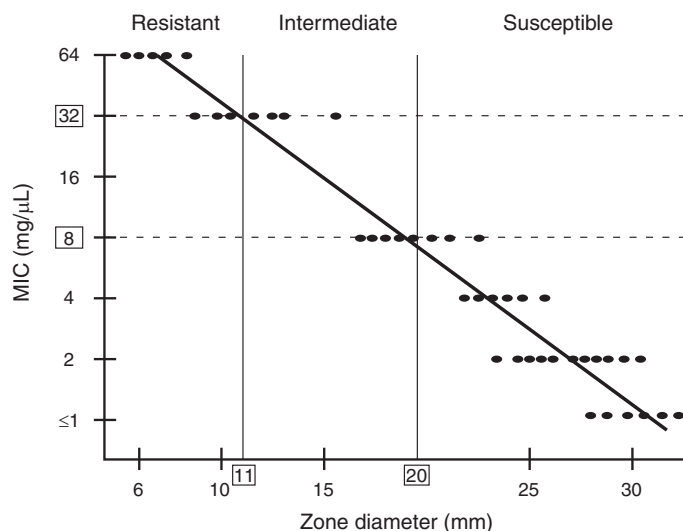


Fig. 13.7 Scattergram and regression analysis plot used to determine disk diffusion zone diameter interpretive breakpoints for hypothetical drug X. Based on clinical response data, isolates with a minimal inhibitory concentration (MIC) of $8.0 \mu\text{g/mL}$ or less are considered susceptible. As derived from this scattergram, corresponding zones of 20 mm or more would be interpreted as susceptible. Isolates with MICs of $32.0 \mu\text{g/mL}$ or more and zones of 11 mm or less are resistant. The intermediate designation is used for isolates whose values fall between the susceptible and the resistant MIC ($16 \mu\text{g/mL}$) and zone interpretive breakpoint (12 to 19 mm).

as other than susceptible (nonsusceptible) when only susceptible or nonsusceptible criteria are specified for the particular antimicrobial agent–organism combination.

Test performance

Most clinical laboratories follow the protocol specified by the CLSI for disk diffusion testing. The CLSI document contains explicit details for test performance.

Disk storage

Disks must be stored properly to ensure that the drugs maintain their potency. For long-term storage, disks are stored at -20°C or below in a non-frost-free freezer. A working supply of disks can be stored in a refrigerator at 2° to 8°C for at least 1 week. Disks should always be stored in a tightly sealed container with desiccant. The container should be allowed to warm to room temperature before it is opened to prevent condensation from forming on the disks when warm room air contacts the cold disks. Only FDA-cleared disks should be used.

Inoculation and incubation

Inoculum suspensions are prepared using a log phase or direct colony suspension to match the turbidity of a McFarland 0.5 standard, as described earlier. A sterile swab is dipped into the suspension, pressed and rotated firmly against the side of the tube to express excess liquid, and then swabbed evenly across the surface of a Mueller-Hinton agar plate. The plate should be swabbed two more times, turning the plate 60 degrees each time, using the same swab (*without* going back into the suspension) to ensure an even “lawn” of bacteria on the plate. Commercially manufactured plate inoculators are available as well (e.g., bioMérieux, Durham, NC). Usually, a plate 150 mm in diameter is used; it can accommodate testing of as many as 12 different antimicrobial disks with most bacteria; placement of more than 12 disks on the plate can result in overlapping zones, which are difficult to measure and may produce erroneous results. At the same time, it is recommended that a “purity plate” (usually sheep blood agar, or chocolate agar for more fastidious organisms) be inoculated from the swab so the next day when results are checked, it can be visually determined with certainty that the isolate was pure on the lawn plate.

Within 15 minutes of inoculation, the antimicrobial disks are applied to the agar individually with sterile forceps or with a multiple-disk dispenser (Fig. 13.8). The disks are pressed firmly to ensure contact with the agar. Within 15 minutes of disk placement, the plates are inverted and placed in a 35°C ambient air incubator for 16 to 18 hours. Although incubation in an atmosphere of increased carbon dioxide (CO_2) is recommended for testing some fastidious bacteria, this should not be done with most organisms. Incubation in CO_2 results in a decreased pH, which affects the activity of some antimicrobial agents.

Reading plates

After incubation, the purity plate is visually inspected to confirm a pure culture was tested. QC plates are read before reading results of patient isolates to determine if the test was performed correctly. The test plate is examined to ensure that the organism grew satisfactorily. The lawn of growth must



Fig. 13.8 Cartridges containing antimicrobial susceptibility test disks are inserted into the dispenser (left). The dispenser (which can hold up to 12 different cartridges of disks) is positioned over an inoculated plate, and light pressure is applied to the handle to simultaneously deposit one of each type of disk onto the plate. The tight-sealing container in the background contains a desiccant packet and is used for storage (at 2° to 8° C) of the dispenser containing a working supply of disks.

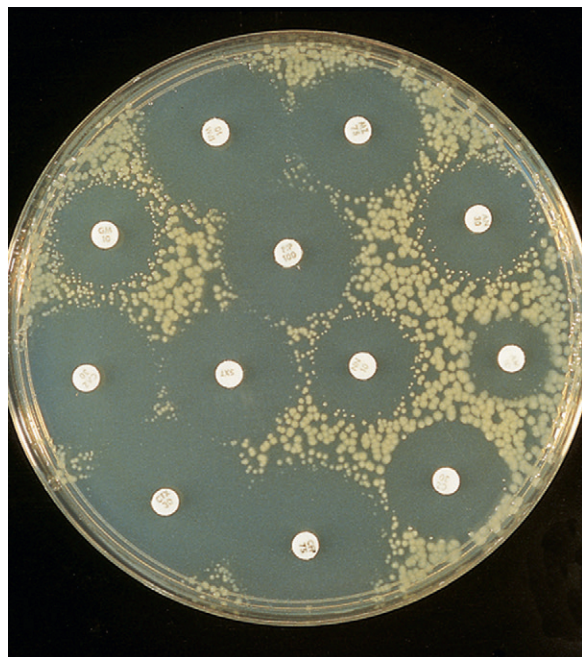


Fig. 13.9 *Escherichia coli* tested by the disk diffusion method. The lawn of growth following overnight incubation shows individual colonies, representing unsatisfactory growth. The most likely explanation for the scanty growth is the use of an inoculum that is too light or contains too many nonviable cells, resulting in larger than normal zones and potentially false-susceptible results.

be confluent or almost confluent. The appearance of individual colonies is unacceptable (Fig. 13.9). If growth is satisfactory, the diameter of each inhibition zone is measured using a ruler, or preferably calipers. Plates are placed a few inches above a black, nonreflecting surface, and zones are examined from the back side (agar side) of the plate illuminated with reflected light (Fig. 13.10). Tiny colonies at the zone edge and

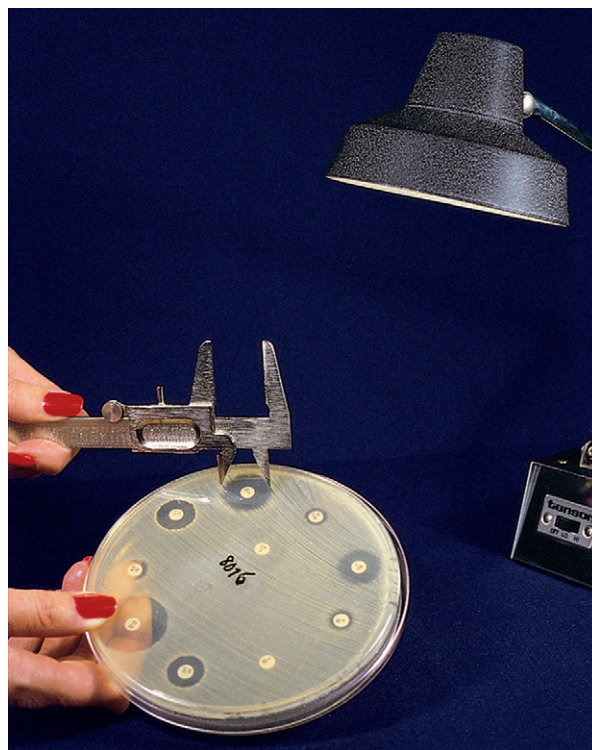


Fig. 13.10 Routine disk diffusion tests are examined by placing the plate on or 2 to 3 inches above a black nonreflecting surface. Reflected light is used to illuminate the plate.

swarm of growth into the zone that often occurs with swarming *Proteus* spp. are ignored; the obvious zone is measured.

As with dilution tests, the end point for the sulfonamides, trimethoprim, and trimethoprim-sulfamethoxazole is an 80% reduction of growth. Obvious colonies within a clear zone should not be ignored. These colonies may occur because of contamination or testing of a mixed culture; however, these colonies sometimes represent a minority resistant subpopulation. When such colonies are noted, the original isolate should be retested. If repeated testing produces the same results, the isolate should be reported as resistant.

Transmitted light (plate held up to a light source; Fig. 13.11) rather than reflected light will increase the accuracy of tests with the **penicillinase-resistant penicillins** linezolid and vancomycin when testing staphylococci and for vancomycin when testing enterococci. Automated disk diffusion zone reading devices, such as BIOMIC (Giles Scientific USA, Santa Barbara, CA), that recognize disk codes and interpret zones digitally are available. The advantages of these devices include speed of analysis in high-volume laboratories and the capacity to store QC and patient isolate images.

Once zone measurements have been made, the millimeter reading for each antimicrobial agent is compared with that specified in the interpretive tables of the CLSI documents or FDA drug package insert, and results are interpreted as susceptible, nonsusceptible, intermediate, or resistant. An excerpt from the chart is shown in Table 13.3. The equivalent MIC breakpoints that are used to define resistance and susceptibility are also shown. Note that as with MIC interpretive criteria, several sets of interpretive criteria that are specific for various organisms or organism groups may exist for some antimicrobial agents.



Fig. 13.11 Disk diffusion tests for staphylococci with oxacillin and vancomycin and for enterococci with vancomycin are examined by holding the plate up to a light source (transmitted light) for zone examination. Any growth within the zone is significant.

Interpretation of in vitro antimicrobial susceptibility test results

Several elements are important when performing in vitro antimicrobial susceptibility tests. A summary of the variables that must be carefully controlled in the performance of disk diffusion and broth microdilution MIC tests is given in Table 13.4. The procedural steps of each method must be followed explicitly to obtain reproducible results. A susceptibility test should never be performed using an inoculum that is not standardized or a mixed culture. For this reason, direct susceptibility tests that incorporate the use of a patient's infected body fluid cannot be recommended, even to obtain a presumptive result. Direct detection of drug resistant genes is a much better option.

The results of a susceptibility test must be interpreted in the laboratory before a report is communicated to a patient's physician. With a disk diffusion test, the inhibition zone size must be interpreted using a table of values that relates the diameter of the zone of inhibition to a category of susceptibility, which is sometimes related to the identity of the isolate. The table used for such interpretations must represent the most recent criteria that have been established by the CLSI. It is important to recognize that the CLSI documents are updated frequently, often annually. Use of old or outdated CLSI tables could represent a serious shortcoming in patient care.

The inhibition zone size and MIC interpretive criteria published by the CLSI and FDA are established by careful

Table 13.3 Zone diameter interpretive standards and equivalent minimal inhibitory concentration breakpoints for several organism groups

Antimicrobial agent	Disk content (μg)	Zone diameter (nearest whole mm)			Equivalent MIC breakpoints (μg/mL)	
		Resistant	Intermediate	Susceptible	Resistant	Susceptible
Ampicillin						
When testing Enterobacterales	10	≤13	14–16	≥17	≥32	≤8
When testing enterococci	10	≤16	—	≥17	≥16	≤8
When testing <i>Haemophilus</i> spp.	10	≤18	19–21	≥22	≥4	≤1
When testing β-hemolytic streptococci	10	—	—	≥24	—	≤0.25
Gentamicin						
When testing Enterobacterales	10	≤12	13–14	≥15	≥16	≤4
When testing <i>Pseudomonas aeruginosa</i>	10	≤12	13–14	≥15	≥16	≤4
When testing staphylococci	10	≤12	13–14	≥15	≥16	≤4
Oxacillin						
When testing <i>Staphylococcus aureus</i>	30 Surrogate: cefoxitin	<21		>22	≥4 (ox) >8 (cefoxitin)	≤2 (ox) <4 (cefoxitin)
When testing <i>Streptococcus pneumoniae</i> (nonpneumonia isolates) for penicillin susceptibility	1		—	≥20	—	≤0.06

MIC, Minimal inhibitory concentration.

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Table 13.4 Primary variables that must be controlled in performance of routine disk diffusion and broth microdilution minimal inhibitory concentration tests

Variable	Standard	Comments
Inoculum	Disk diffusion: 1.5×10^8 CFU/mL Broth microdilution: 5×10^5 CFU/mL (final concentration)	Use "adequate" McFarland turbidity standard (0.5 for disk diffusion) When preparing direct suspensions (without incubation), do not use growth from plates >1 day old
Media		
Formulation	Mueller-Hinton	Prepare in house or purchase from reliable source Perform media quality control to verify acceptability before use for patient tests
Ca ²⁺ , Mg ²⁺ content	25 mg/L Ca ²⁺ , 12.5 mg/L Mg ²⁺	Increased concentrations result in decreased activity of aminoglycosides against <i>Pseudomonas aeruginosa</i> and decreased activity of tetracyclines against all organisms (decreased concentrations have the opposite effect)
Thymidine content	Minimal or absent	Excessive concentrations can result in false resistance to sulfonamides and trimethoprim
pH	7.2–7.4	Decreased pH can lead to decreased activity of aminoglycosides, erythromycin, and clindamycin and increased activity of tetracyclines (increased pH has the opposite effect)
Agar depth (disk diffusion)	3–5 mm	Possibility for false susceptibility if <3 mm or false resistance if >5 mm
Incubation		
Atmosphere	Humidified ambient air	CO ₂ incubation decreases pH, which can lead to decreased activity of aminoglycosides, erythromycin, and clindamycin and increased activity of tetracyclines
Temperature	35° C	Some MRSA may go undetected if >35° C
Length	Disk diffusion: 16–18 hours	Some MSRA may go undetected if <24 hours
	Broth microdilution: 16–20 hours	Some vancomycin-resistant enterococci may go undetected if <2 hours with disk diffusion
	24 hours for staphylococci with oxacillin ^a and vancomycin and for enterococci with vancomycin and gentamicin HLAR; 48 hours for enterococci with streptomycin HLAR; 24 hours sometimes needed for fastidious bacteria	Some HLAR (gentamicin) enterococci may go undetected if <24 hours (broth microdilution) Some HLAR (streptomycin) enterococci may go undetected if <48 hours (broth microdilution)
Antimicrobial agents		
Disks	Use disks containing appropriate FDA- or CLSI-defined concentration of drug	Check CLSI publication or FDA package insert (accompanying disks) for specifications.
	Proper storage	For long-term storage, use non-frost-free freezer at –20° C or less in a tightly sealed, desiccated container For short-term storage (at least 1 week), maintain temperature at 2° to 8° C in a tightly sealed, desiccated container Allow to warm to room temperature before opening container
	Proper placement on agar	Place 12 or fewer disks/150-mm plate (no overlapping zones)
Solutions	Prepared from reference standard powders	Pharmacy-grade antimicrobial agents unacceptable (may not show antimicrobial activity in vitro)
	Proper storage	Store in non-frost-free freezer, optimally at –70° C or less Never refreeze
End point measurement		
Disk diffusion	Reflected light (except for staphylococci with oxacillin ^a and vancomycin, and for enterococci with vancomycin) and plate held against black background	Lawn must be confluent or almost confluent Ignore faint growth of tiny colonies at zone edge
	Zones measured from back of plate	Trimethoprim and sulfonamide end point at 80% or more inhibition Ignore swarm within obvious zone for swarming <i>Proteus</i> spp Retest when colonies are within zone (except for staphylococci with oxacillin, and enterococci with vancomycin)
	Transmitted light used for staphylococci with oxacillin ^a and vancomycin, and for enterococci with vancomycin	Call <i>resistant</i> if any growth within zone (unless possibly artifactual or contaminated) Reproducibility of zone measurements is within ± 2 mm

Continued

Table 13.4 Primary variables that must be controlled in performance of routine disk diffusion and broth microdilution minimal inhibitory concentration tests—Cont'd

Variable	Standard	Comments
Broth microdilution	Adequate lighting and reading device	MIC is the lowest concentration that inhibits growth (turbidity, haze, or pellet) Sulfonamides and trimethoprim may trail (ignore trailing <2-mm buttons) Justify "skip wells" or repeat Staphylococci and penicillin: perform induced β -lactamase test if MIC = 0.06–0.12 μ g/mL Reproducibility should be within ± 1 twofold dilution
^a Includes all penicillinase-resistant penicillins (oxacillin, methicillin, nafcillin, and dicloxacillin). CFU, Colony-forming units; CLSI, Clinical and Laboratory Standards Institute; FDA, U.S. Food and Drug Administration; HLAR, high-level aminoglycoside resistance; MIC, minimal inhibitory concentration; MRSA, methicillin-resistant <i>Staphylococcus aureus</i> . Modified from Hindler, J. A., & Mann, L. M. (1992). Principles and practices for the laboratory guidance of antimicrobial therapy. In R. C. Tilton, et al. (Eds.), <i>Clinical laboratory medicine</i> . St. Louis: Mosby.		

analysis of three types of data: (1) microbiological data (e.g., a comparison of MICs vs. zone sizes on a large number of bacterial strains, including those with known resistance mechanisms); (2) pharmacokinetic data (e.g., serum, cerebrospinal fluid [CSF], urine, and other body fluids and tissue levels of an antimicrobial agent); and (3) results of clinical studies obtained before FDA approval and marketing of an antimicrobial agent. Thus MIC interpretive criteria are not based simply on a comparison of serum levels of an antimicrobial agent and MICs. Zone diameter interpretive criteria are, however, based largely on direct correlations of MICs and zone sizes.

Whether based on the determination of an MIC or on interpretation of a disk diffusion zone diameter, the four categories of susceptibility should be interpreted in the same manner. If the MIC or zone size is interpreted as susceptible using the most recent interpretive criteria, the clinical interpretation of the result is that the patient's infecting organism should respond to therapy with that antimicrobial agent using the recommended dosage for the site of infection. Conversely, an MIC or zone size interpreted as resistant is unlikely inhibit the bacterium by the usually achievable concentrations of the antimicrobial agent based on the normal dosages used with that drug. An intermediate result indicates that a bacterium falls into a range of susceptibility in which the MIC approaches or exceeds the level of antimicrobial agent that can ordinarily be achieved, and for which clinical response is likely to be less than with a susceptible strain. Exceptions can occur if the antimicrobial agent is highly concentrated in a body fluid, such as urine, or if a higher-than-normal dosage of the antimicrobial agent can be safely administered (e.g., some penicillins and cephalosporins). At times, the intermediate result means that certain variables in the susceptibility test may not have been well controlled, and the values have fallen into a zone, separating susceptible from resistant strains.

Another category that may be used for reporting is *non-susceptible*. This category is used when there are no intermediate or resistant interpretive criteria, only a susceptible interpretive criterion, and the MIC or disk diffusion zone size for classifying the organism as susceptible is not achieved. In these cases, the identity of the organism should be confirmed and susceptibility testing repeated. If the results are still nonsusceptible, it may be appropriate to send the bacterium to a reference laboratory. The nonsusceptible category commonly occurs with newer antimicrobials because during

drug development and initial clinical trials, no organisms were found that were resistant to the agent. The category nonsusceptible does not necessarily mean that the organism is resistant to the antimicrobial.

Certain other specific aspects of susceptibility test reporting are detailed in the CLSI tables, such as refraining from reporting results for antimicrobial agents that do not enter the CSF on isolates from patients who have meningitis. Also, results for antimicrobial agents that are only useful for treating UTIs must not be reported on isolates from specimens other than urine.

Modified test methods for slow-growing or fastidious bacteria

Mueller-Hinton broth and agar are the standard media used for routine dilution and disk diffusion susceptibility tests. These media, however, do not support the growth of all bacteria that require testing; consequently, routine methods must be modified for testing fastidious bacteria that require supplemental nutrients, modified incubation conditions, or both.

Streptococcus species

Streptococcus spp., including *Streptococcus pneumoniae*, require a more nutritious medium for growth; they will not grow satisfactorily on unsupplemented Mueller-Hinton medium. Broth dilution tests are performed in Mueller-Hinton broth that has been supplemented with 2% to 5% lysed horse blood. The standard disk diffusion procedure uses Mueller-Hinton agar supplemented with 5% sheep blood incubated in 5% CO₂ for 20 to 24 hours. Tests performed on media containing blood are examined from the top of the plate with the lid removed. For plates containing blood, it is important to read the zone of growth inhibition and not the zone of hemolysis.

Penicillins and cephalosporins remain the drugs of choice for treating pneumococcal infections; however, resistance to penicillin and to other potentially useful agents is widespread. Penicillin resistance in *S. pneumoniae* is caused by the presence of altered penicillin-binding proteins (drug targets in the cell wall). It is possible to screen for penicillin susceptibility in *S. pneumoniae* using an oxacillin disk (1 μ g), except on isolates from blood or CSF, in which case penicillin MIC

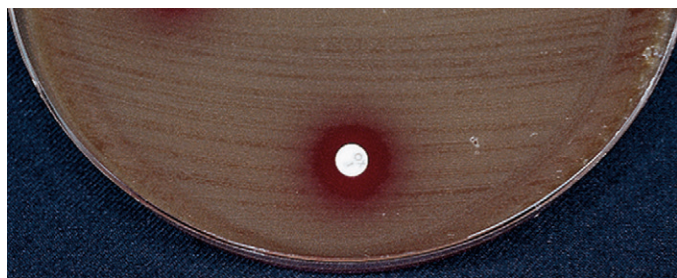


Fig. 13.12 An oxacillin (1- μ g) disk is used to screen for penicillin susceptibility in *Streptococcus pneumoniae* isolated from specimens other than cerebrospinal fluid and blood. If the oxacillin zone of inhibition is 20 mm or larger from a non meningitis isolate, it is reported as penicillin susceptible. If the oxacillin zone is 19 mm or smaller, a penicillin minimal inhibitory concentration (MIC) test must be performed. The isolate pictured has an oxacillin zone of approximately 15 mm, so a penicillin MIC test must be performed.

testing must be performed. A penicillin disk should not be used for screening purposes because it is less accurate. If the oxacillin zone of inhibition is 20 mm or larger, the isolate can be safely reported as penicillin (not oxacillin) susceptible (penicillin MIC $\leq 0.06 \mu\text{g/mL}$). If the oxacillin zone is 19 mm or smaller, a penicillin (not oxacillin) MIC test must be performed to determine the degree of resistance (Fig. 13.12).

S. pneumoniae isolates from non-CSF sites with penicillin MICs of $0.06 \mu\text{g/mL}$ or less are interpreted as susceptible, with penicillin MICs of 0.12 to $1.0 \mu\text{g/mL}$ as intermediate, and with penicillin MICs of $2.0 \mu\text{g/mL}$ or more as resistant when orally administered penicillin V is to be used for treatment. When parenterally administered penicillin is to be used for treatment of isolates from non-CSF sources (e.g., from sputum), the isolate is considered susceptible when the MIC is $2 \mu\text{g/mL}$ or less, intermediate when the MIC is $4 \mu\text{g/mL}$, and resistant when the MIC is $8 \mu\text{g/mL}$ or more. If the *S. pneumoniae* isolate is from CSF, the isolate is considered susceptible to penicillin when the MIC is $0.06 \mu\text{g/mL}$ or less and resistant when the MIC is $0.12 \mu\text{g/mL}$ or more. Determining the degree of penicillin resistance is important because the recommended therapy may be different for penicillin-intermediate and penicillin-resistant strains. It is important to test penicillin-resistant isolates with other clinically relevant antimicrobial agents, such as cefotaxime or ceftriaxone (by an MIC method, not disk diffusion), erythromycin, a fluoroquinolone, vancomycin, and perhaps clindamycin or a tetracycline.

The methods for testing nonpneumococcal streptococci are the same as those for *S. pneumoniae*; however, separate breakpoints for this group of bacteria have been defined. β -hemolytic streptococci remain universally susceptible to penicillin, the drug of choice for treating infections caused by these organisms; therefore routine susceptibility testing on β -hemolytic streptococci is generally not necessary. For penicillin-allergic patients, alternative agents for therapy include a macrolide (e.g., erythromycin) or clindamycin. In contrast with penicillin, some β -hemolytic streptococci are resistant to erythromycin only or to erythromycin and clindamycin. Isolates that appear erythromycin-resistant and clindamycin-susceptible may have inducible or constitutive resistance to clindamycin. Before reporting clindamycin as susceptible in these cases, a **D-zone test** must be performed, as described later in this chapter for staphylococci.

Accurate penicillin susceptibility results are needed for viridans streptococci isolated from serious infections such as bacteremia or endocarditis. If the isolate has a penicillin MIC of $0.12 \mu\text{g/mL}$ or less, penicillin alone is often prescribed; however, higher penicillin MICs (0.25 to $2.0 \mu\text{g/mL}$) suggest the need for concomitant therapy with an aminoglycoside. Isolates with penicillin MICs greater than $2.0 \mu\text{g/mL}$ are highly resistant, and for these isolates, vancomycin rather than penicillin is generally prescribed. Because of the critical nature of penicillin susceptibility results, the disk diffusion test is not recommended; instead, MIC tests should be performed. As with *S. pneumoniae*, penicillin resistance in viridans streptococci is caused by altered penicillin-binding proteins.

Haemophilus influenzae and Haemophilus parainfluenzae

Haemophilus test medium (HTM), which consists of Mueller-Hinton base supplemented with X (hematin) and V (nicotinamide adenine dinucleotide [NAD]) factors, has been standardized for testing *Haemophilus influenzae* and *Haemophilus parainfluenzae*. HTM broth is used for broth dilution tests, and HTM agar is used for disk diffusion tests. The procedures for *Haemophilus* spp. are identical to those described for nonfastidious bacteria, with the exception that the disk diffusion test with HTM is incubated in an atmosphere of 5% to 7% CO_2 . Zone diameter and MIC interpretive criteria unique for this genus are available. For some agents, such as cefotaxime, only a susceptible range is defined because cefotaxime-resistant *Haemophilus* spp. have not been identified. In cases in which the determined MIC is greater than what would make the organism cefotaxime-susceptible, the organism is reported as nonsusceptible. In these cases, the organism should be reidentified and the MIC redetermined. If the same results are obtained after retesting, the organism should be submitted to a reference laboratory, such as a state health laboratory.

Ampicillin or amoxicillin is often effective in treating localized, less serious *H. influenzae* infections; however, 25% to 50% of *H. influenzae* isolates produce a β -lactamase that inactivates these agents. β -Lactamase-producing isolates can be quickly identified by using a rapid β -lactamase bench test. *H. influenzae* also may be resistant to ampicillin and amoxicillin because of altered penicillin-binding proteins. These isolates are referred to as β -lactamase-negative, ampicillin-resistant (BLNAR). This resistance occurs in less than 1.0% of clinical isolates in the United States but is higher in other countries, such as Japan. Very small proportions of isolates possess resistance caused by altered penicillin-binding proteins and production of β -lactamase and are referred to as β -lactamase-positive, amoxicillin-clavulanate-resistant (BLPACR) isolates. The BLNAR and BLPACR isolates are both detectable only by in vitro susceptibility testing. There are no rapid detection methods for these types of resistance. Because most ampicillin-resistant (amoxicillin-resistant) *H. influenzae* isolates produce β -lactamase, and *H. influenzae* is often susceptible to alternative agents currently recommended, some laboratories only perform a β -lactamase test or test only ampicillin and trimethoprim-sulfamethoxazole by the disk diffusion or MIC method. However, they will test additional agents (e.g., fluoroquinolones) if the isolate is from nonrespiratory sources or if requested by clinicians.

Neisseria gonorrhoeae and *Neisseria meningitidis*

Neisseria gonorrhoeae and *Neisseria meningitidis* are of public health significance. Therapy for disseminated meningococcal infections and various types of gonococcal infections is generally empiric, based on recommendations from the Centers for Disease Control and Prevention (CDC) and various professional groups. Usually, clinical microbiology laboratories are not required to perform antimicrobial susceptibility testing of these two species. Public health laboratories, however, may receive requests to test them on a periodic basis. The CLSI has described MIC and disk diffusion methods for these species.

Penicillin was the drug of choice for treating uncomplicated gonorrhea. However, penicillin-resistant isolates emerged in the 1970s, and fluoroquinolone-resistant isolates emerged in the 1990s. This led to the current use of ceftriaxone or cefixime as first-line therapy. Penicillin resistance in *N. gonorrhoeae* can be caused by the production of a β -lactamase like that produced by ampicillin-resistant *H. influenzae*, and this resistance can be readily detected with a rapid β -lactamase test. β -Lactamase-producing isolates are referred to as **penicillinase-producing *Neisseria gonorrhoeae* (PPNG)**. Some *N. gonorrhoeae* isolates are penicillin resistant because of an altered penicillin-binding protein; this resistance can be detected only with conventional dilution or disk diffusion tests. The production of altered penicillin-binding proteins is chromosomally mediated, and *N. gonorrhoeae* with altered penicillin-binding proteins is often referred to as **chromosomally mediated resistant *Neisseria gonorrhoeae* (CMRNG)**.

Because of continuing changes in the susceptibility of *N. gonorrhoeae*, the need for testing the susceptibility of isolates in hospital laboratories may be necessary again after a hiatus for several years. Since the implementation and widespread use of rapid nucleic acid testing methods for *N. gonorrhoeae*, however, fewer isolates are grown in culture, making it difficult, unfortunately, to monitor these changes.

Gonococcal agar base is supplemented with various nutrients for testing *N. gonorrhoeae*. Dilution tests are performed using agar dilution because this species tends to lyse in broth media, resulting in false-susceptible results. Disk diffusion tests are performed on the same agar, and all tests are incubated in an atmosphere containing 5% to 7% CO₂. The CLSI and FDA have specified interpretive criteria unique for this species. For several agents, resistant isolates have not yet been encountered, so only susceptible criteria are available.

Except for very rare isolates that produce β -lactamase, *N. meningitidis* is generally susceptible to penicillin; however, in the United States, ceftriaxone or cefotaxime is usually the drug of choice for treating invasive meningococcal infections. Isolates with elevated penicillin MICs (penicillin-intermediate) have been reported, although their significance is minimized through the extensive use of third-generation cephalosporins for therapy. Meningococci are often resistant to sulfonamides (including trimethoprim-sulfamethoxazole) and are occasionally resistant to rifampin. For these reasons, a fluoroquinolone (e.g., ciprofloxacin) is usually administered prophylactically to individuals in close contact with patients with meningococcal meningitis. The CLSI has described MIC and disk diffusion methods for testing meningococci that are the same as those used for testing the streptococci, except that broth microdilution tests with meningococci require CO₂

incubation. Breakpoints for determining susceptibility or resistance in meningococci for several agents can be found in the latest CLSI tables.

Helicobacter pylori and *Campylobacter* species

The susceptibility of *H. pylori* to antimicrobials is best determined by agar dilution testing using Mueller-Hinton agar containing 5% aged (≥ 2 weeks old) sheep blood. Susceptibility test plates must be incubated in a microaerobic environment and read at 3 days of incubation. Genotypic tests in development for identification of the organism and resistance genes using polymerase chain reaction (PCR) assays and sequencing techniques directly from the specimen show promising results. *Campylobacter* spp. can also be tested using agar dilution, or broth microdilution and disk diffusion methods. CLSI guidelines detail specific procedures for testing for both genera, as well as the QC strains to use and interpretive criteria for specific antimicrobials.

Susceptibility testing of agents of bioterrorism

With the occurrence of the terrorist attacks in the United States on September 11, 2001, the more imminent threat of the use of bacteria and other pathogens as agents of bioterrorism was given heightened attention. Before this event, there were no standardized methods for testing the susceptibility of *Bacillus anthracis*, *Yersinia pestis*, *Burkholderia mallei*, *Burkholderia pseudomallei*, *Francisella tularensis*, and *Brucella* spp. to antimicrobials. In addition, knowledge was limited regarding the use of antimicrobials to prevent or treat infections caused by these bacteria. Since then, the CLSI has standardized the methods for susceptibility testing of these bacteria against a variety of antimicrobials and has established interpretive criteria for the results of these tests (publication M45). In addition, a great deal more information is now available on the use of antimicrobials that can be used to prevent infections with these organisms and to treat infections if they do occur. However, these organisms are an imminent danger when being tested in the laboratory, and work with these organisms should be conducted only in a biosafety level 2 (BSL2) or higher facility by trained individuals. This limitation applies even to routine susceptibility tests. As soon as it is suspected that a specimen may contain one of these agents or that one of these bacteria has been isolated, the specimen or isolate must be quarantined and sent to a state health laboratory after state health officials are alerted. Clinical laboratories should establish contingency plans to deal with situations in which bioterrorism agents have appeared, or potentially appeared, in the laboratory, especially if a staff member has been exposed and might need prophylaxis.

Obligate anaerobic bacteria

The reference method described by the CLSI for testing anaerobic bacteria is an agar dilution method, and the recommended medium is supplemented *Brucella* laked sheep blood agar. As noted, however, agar dilution is not practical for use in the routine clinical laboratory; a broth microdilution method is used more often. The CLSI broth microdilution procedure resembles that used for testing aerobes, except that *Brucella* broth with lysed horse blood is used for testing the

B. fragilis group of anaerobes. Also, the number of organisms in the test inoculum is $0.5 \log_{10}$ higher (10^6 CFU/mL) than that for testing aerobes, and panels are incubated anaerobically at 35° C for 48 hours. As with tests for aerobic bacteria, the CLSI has defined *susceptible*, *intermediate*, and *resistant* criteria for the interpretation of MICs for anaerobes. Several commercial companies produce broth microdilution MIC panels for testing anaerobes. The **Etest** (AB Biodisk, Solna, Sweden), discussed in detail later, has been shown to perform satisfactorily for susceptibility testing of anaerobes and is used in many clinical laboratories.

Infrequently encountered or fastidious bacteria

Besides anaerobes, the CLSI publication M45 focuses on infrequently encountered or fastidious bacteria not addressed in the standard MIC and disk diffusion documents. When clinically indicated, isolates of corynebacteria (and some other gram-positive bacilli), *Aeromonas*, *Vibrio*, *Pasteurella*, *Moraxella catarrhalis*, some members of the HACEK (*Haemophilus* spp., *Aggregatibacter* spp., *Cardiobacterium* spp., *Eikenella corrodens*, and *Kingella* spp.) group of gram-negative bacteria, and *Abiotrophia* and *Granulicatella* can be tested using various standard CLSI methods; the results are interpreted using the CLSI tables specific for each organism. CLSI publication M24-A2 (2011) offers guidelines for antimicrobial testing of acid-fast bacilli, including *Mycobacterium tuberculosis*, nontuberculous mycobacteria (NTM), and *Nocardia*. Yeasts and filamentous fungi can be tested using agar dilution, standardized disk diffusion and Etest tests, broth macrodilution, and microdilution techniques. Some commercial automated systems can perform antifungal susceptibility testing of *Candida* spp. as well.

Additional organism and antimicrobial agent testing concerns

Special procedures must be used to detect clinically significant resistance in some nonfastidious bacteria.

Case check 13.2

Laboratories' procedures include ways of detecting drug resistance in all clinically significant microorganisms. The method must be determined to provide an accurate and reproducible result. It is important to know which assay to use and if modifications are necessary for unusual or fastidious isolates.

Detection of oxacillin (methicillin) resistance in staphylococci

Oxacillin and other penicillinase-resistant penicillins, such as methicillin, nafcillin, cloxacillin, and dicloxacillin, constitute the drug class of choice for treating staphylococcal infections. Oxacillin is the class representative generally used to detect resistance in staphylococci and has produced more reliable results than testing any of the other agents. When an isolate shows resistance to one of the penicillinase-resistant penicillins, it must be considered resistant to the entire group. Staphylococcal resistance to the penicillinase-resistant

penicillins is most often caused by the presence of a unique penicillin-binding protein (PBP2a or PBP2') in the cell wall. The *mecA* gene encodes an altered penicillin-binding protein, which has a low affinity for binding all β -lactam drugs. In addition, oxacillin resistance in staphylococci can be caused by the *mecC* gene, which has been recovered from isolates from livestock and humans with animal exposure, although the prevalence is currently low. The *mecC* gene has about 70% homology to *mecA* and codes for a PBP2a protein with only about 63% similarity to the *mecA* product. Therefore the PCR assay for *mecA* and the antigen tests for PBP2a will not detect *mecC* oxacillin resistance.

Although methicillin is no longer available, isolates of oxacillin-resistant *S. aureus* are commonly referred to as MRSA for historic reasons. Because some staphylococci exhibit heteroresistance or heterogeneous expression of resistance to oxacillin, detecting oxacillin resistance in isolates that possess the *mecA* gene has sometimes proven difficult under standard susceptibility testing conditions. In **heteroresistant** isolates, all cells in the population have the genetic element (*mecA* gene) for oxacillin resistance, but not all the cells express this resistance by virtue of PBP2a production. Consequently, in the oxacillin susceptibility test, some cells may appear resistant, and some can appear susceptible. If too few cells appear resistant, an oxacillin-resistant strain might not be detected. In addition, a rapid (5-minute) PBP2a colorimetric bench test, such as the Clearview PBP2a SA Culture Colony Test (Abbott Laboratories, Chicago, IL), may be performed on the isolate. However, this is not 100% reliable in detecting oxacillin resistance.

In vitro testing conditions can be modified to enhance the expression of oxacillin resistance, as follows:

- Preparation of inocula using the direct inoculum suspension procedure
- Incubation of tests at temperatures no higher than 35° C
- Making final test readings after a full 24 hours of incubation
- Supplementation of Mueller-Hinton broth or agar with 2% NaCl for dilution tests

The extended incubation allows the more slowly growing resistant subpopulation sufficient time to grow to detectable numbers. In addition, test plates should always be examined very closely. For oxacillin disk diffusion tests, zones of inhibition must be examined by using transmitted light (holding the plate up to the light source; see Fig. 13.11), and any growth is considered significant. A haze of growth within the inhibition zone for oxacillin-resistant isolates is sometimes observed (Fig. 13.13).

An **oxacillin-salt screen plate** that contains Mueller-Hinton agar supplemented with 4% NaCl and 6 μ g/mL oxacillin has been used to detect MRSA. The high salt concentration makes the medium selective for staphylococci. To perform the oxacillin screen test, a McFarland 0.5 suspension is prepared. A swab is dipped into this suspension and streaked over an area of approximately 2 $\times$ 5 cm or deposited as a spot on the agar surface. After overnight incubation at 35° C, growth (more than one colony) is an indication that the isolate is oxacillin resistant. This method does not reliably detect oxacillin-resistant, coagulase-negative staphylococci.

Testing a surrogate marker of resistance (i.e., cefoxitin) may provide a more accurate indication of oxacillin resistance

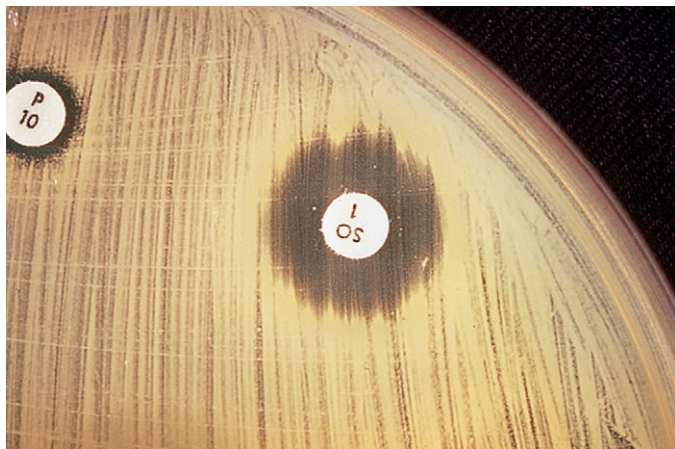


Fig. 13.13 The oxacillin zone for heteroresistant oxacillin-resistant *Staphylococcus aureus* often shows a haze of growth within the zone of inhibition. This haze is significant, and the isolate here is oxacillin resistant.

than testing oxacillin itself. This is because cefoxitin induces greater expression of PBP2a in *mecA*-containing strains of staphylococci and functions as a test agent to detect resistance. Studies have shown that performing a disk diffusion test with cefoxitin and using the specific cefoxitin breakpoints recommended by the CLSI provides sensitivity and specificity equivalent to testing oxacillin (by disk diffusion and MIC methods) with *S. aureus* and greater sensitivity and specificity of cefoxitin when testing coagulase-negative staphylococci. With *S. aureus*, cefoxitin disk diffusion test results are more easily interpreted than the results of disk diffusion tests using oxacillin. Thus cefoxitin disk diffusion testing is now recommended by the CLSI as the preferred method for detection of oxacillin resistance in *S. aureus* and coagulase-negative staphylococci. Even though it is coagulase-negative, *Staphylococcus lugdunensis* has characteristics that resemble those of *S. aureus*, and the results of the cefoxitin screen test should be interpreted using *S. aureus* interpretive criteria. It is important to report the findings from the cefoxitin disk diffusion test as indicative of oxacillin susceptibility or resistance; cefoxitin and cefazolin results should not be reported.

Case check 13.3

Just because a bacterium is susceptible in vitro to an antimicrobial, it does not mean that it can be used to treat an infection caused by the bacterium. In some cases, additional testing is required, as in the case of clindamycin-inducible resistance.

Sometimes, oxacillin-resistant staphylococci can appear susceptible in vitro to other β -lactam agents, such as the cephalosporins; however, these are clinically ineffective. Regardless of the in vitro test results, all oxacillin-resistant staphylococci must be reported as resistant to all β -lactam agents (including cephalosporins, β -lactam- β -lactamase inhibitor combinations, and carbapenems) if those agents are tested. For practicality, it is better simply to perform the cefoxitin disk diffusion test and deduce susceptibility to other β -lactam agents based on whether a staphylococcal isolate is oxacillin-susceptible or oxacillin-resistant. MRSA is a significant

cause of community-associated and healthcare-associated infections and requires that all clinical microbiology laboratories accurately detect drug resistance in *S. aureus*. Previously, healthcare-associated strains of MRSA were often resistant to several other drug classes in addition to the β -lactams. Although recent community-associated MRSA isolates often have been resistant only to the macrolides (e.g., erythromycin) in addition to various β -lactams, an increasing number of MRSA isolates from the community have the same antimicrobial resistance profile as healthcare-associated MRSA isolates.

Rare *S. aureus* isolates have a subtler and less common type of oxacillin resistance unrelated to the presence of the *mecA* or *mecC* genes. The resistance mechanism in these isolates is caused by hyperproduction of β -lactamase, or sometimes the presence of a point mutation in the gene coding for penicillin-binding protein but not PBP2a. These isolates generally have MICs just above (typically 1 to 8 $\mu\text{g}/\text{mL}$) or zones of inhibition just below the breakpoint for oxacillin susceptibility, and they are sometimes referred to as **borderline oxacillin-resistant *S. aureus*** (BORSA). Currently, the CLSI breakpoint for *S. aureus* $<2 \mu\text{g}/\text{mL}$ is sensitive and $>4 \mu\text{g}/\text{mL}$ is resistant; there is no intermediate result. Isolates with borderline oxacillin resistance generally do not grow on oxacillin screening plates. The clinical response of BORSA to penicillinase-resistant penicillins and to other β -lactam agents has not been clearly defined. The exact prevalence of BORSA strains is difficult to establish but is estimated to be 5% of *S. aureus* isolates. One study found that approximately 5% of asymptomatic children carried BORSA in the nares. Antimicrobial administration is likely the main risk factor for developing the BORSA phenotype.

Vancomycin resistance or diminished susceptibility in *Staphylococcus aureus*

Between 2002 and 2005, five different isolates of MRSA with vancomycin resistance were detected for the first time. These isolates had apparently either acquired a plasmid containing the *vanA* vancomycin resistance gene from **vancomycin-resistant enterococci** (VRE) or had developed mutations resulting in their cell walls being less susceptible to vancomycin. The MRSA isolates demonstrated various levels of resistance to vancomycin. Some were obviously resistant by routine susceptibility testing methods, and others were initially missed by routine testing methods. This important resistance followed recognition in 1996 of the first MRSA isolate, with subtle diminished susceptibility to vancomycin. Shortly thereafter, several similar isolates were encountered that all had vancomycin MICs of 8 $\mu\text{g}/\text{mL}$. Isolates of *S. aureus* with reduced susceptibility to vancomycin (MIC 4 to 8 $\mu\text{g}/\text{mL}$) are called **vancomycin-intermediate *Staphylococcus aureus*** (VISA). Resistance is not caused by the *vanA* gene but by changes in the cell wall, rendering them less susceptible to vancomycin. Although still uncommon, VISA and **vancomycin-resistant *Staphylococcus aureus*** (VRSA) isolates (as of 2019, only 14 strains of VRSA have been documented in the United States between 2002 and 2015) are of great concern because vancomycin is one of the most proven agents for treating serious MRSA infections. VRSA is defined as isolates with a vancomycin MIC $\geq 16 \mu\text{g}/\text{mL}$.

The CLSI recommends use of the broth microdilution test or vancomycin agar screen for enterococci as the best methods

for detection of VISA and VRSA. The **vancomycin agar screen plate** contains brain-heart infusion (BHI) agar supplemented with 6 µg/mL vancomycin and is useful in screening for vancomycin resistance in *S. aureus* and enterococci. The vancomycin disk diffusion test has not uniformly detected VISA and VRSA isolates, and some of the commercial susceptibility testing instruments have similarly not always provided reliable detection of these strains. In addition, heteroresistant vancomycin-intermediate *S. aureus* (hVISA) are isolates that normally test as susceptible by standard methods but contain a subpopulation of cells that test as nonsusceptible for vancomycin. The use of the macro Etest method, a modification of the standard Etest discussed later, has proven to be valuable in detecting hVISA. Because the test uses a higher concentration of bacteria (approximately 1×10^8 /mL) than that used routinely for susceptibility testing, the probability of detecting the small number of these organisms in the overall population of cells is enhanced. The Etest method can be used for detecting heteroresistance in other organisms to other antimicrobials.

Inducible clindamycin resistance in staphylococci

Two different mechanisms confer macrolide (e.g., erythromycin) resistance in staphylococci. The *erm* gene codes for methylation of the 23S ribosomal ribonucleic acid (rRNA), which results in resistance to erythromycin and inducible or constitutive resistance to clindamycin. The *msrA* gene codes for an efflux mechanism, which results in resistance to erythromycin but susceptibility to clindamycin. Consequently, when an erythromycin-resistant and clindamycin-susceptible staphylococcal isolate is encountered, a **D-zone test** for inducible clindamycin resistance must be performed before clindamycin is reported as susceptible. For the D-zone test, an erythromycin disk is placed adjacent to a clindamycin disk (15 to 26 mm, edge to edge) as part of a standard disk diffusion test. After overnight incubation, flattening of the clindamycin zone between the two disks (Fig. 13.14) indicates that the isolate has inducible clindamycin resistance because of *erm*. No flattening indicates that the isolate is erythromycin-resistant only (because of *msrA*). When an isolate demonstrates inducible resistance, clindamycin is reported as resistant. The phenomenon of clindamycin-inducible resistance also exists in *S. pyogenes* and *S. pneumoniae* and can be detected in a similar manner. Some automated susceptibility instruments can detect this inducible resistance.

Enterococci

The enterococci express low-level resistance to several antimicrobial agents including the aminoglycosides and β -lactams. In addition, they can also express high-level resistance to several agents. Penicillin resistance among the *Enterococcus* spp. is generally caused by overexpression of PBP with low affinity to the penicillins. Occasionally, *Enterococcus* isolates express a β -lactamase, notably *Enterococcus faecalis* and *Enterococcus faecium*. β -lactamases inactivate β -lactam molecules by disrupting the β -lactam ring component of the molecule (Fig. 13.15). Ampicillin or penicillin is normally effective in treating uncomplicated enterococcal infections (e.g., UTIs) by most *Enterococcus* spp. These cell wall active agents are only bacteriostatic against enterococci, and when administered alone are inadequate for treating serious infections, such as endocarditis, that require bactericidal therapy. To obtain a bactericidal effect, ampicillin or penicillin (or vancomycin in patients with penicillin allergies or with *E. faecium* infection) must be given in combination with an aminoglycoside, usually gentamicin or sometimes streptomycin.

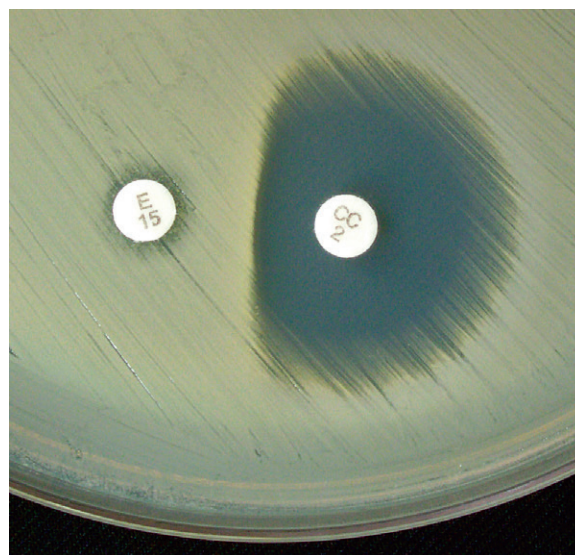


Fig. 13.14 D-zone testing of *Staphylococcus aureus* that has *erm*-mediated inducible clindamycin resistance. The positive reaction is noted by a flattening of the zone around the clindamycin disk in the area in which there has been diffusion of erythromycin and clindamycin molecules overlap.

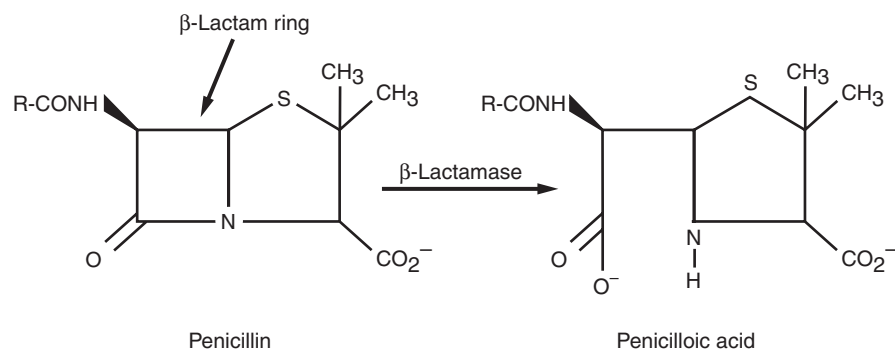


Fig. 13.15 β -Lactamase hydrolyzes the β -lactam ring portion of the penicillin molecule. The hydrolysis results in the formation of penicilloic acid, which does not have antibacterial activity.

Enterococci with the *blaZ* gene constitutively express β -lactamase at a much lower level than *S. aureus*, resulting in a clinically significant inoculum effect. Inoculating bacteria at concentrations for routine susceptibility testing (generally 1×10^5 organisms/mL), enterococci produce so little enzyme that they test susceptible to β -lactam agents. However, at a high inoculum, such as during an infection, the presence of more enzyme leads to resistance. Once the presence of a β -lactamase is identified, the addition of the β -lactamase inhibitor sulbactam (i.e., ampicillin-sulbactam) is sufficient to restore the antibiotic efficacy.

Detection of high-level aminoglycoside resistance in enterococci

Enterococci are inherently resistant to low concentrations of aminoglycosides, precluding their use as single agents for treatment of enterococcal infections. This low-level resistance is caused by poor drug uptake by enterococcal cells. For isolates with low-level aminoglycoside resistance, a synergistic interaction occurs when an aminoglycoside is administered together with a cell wall active agent such as ampicillin, penicillin, or vancomycin. Sometimes, however, enterococci develop **high-level aminoglycoside resistance** in which the aminoglycoside does not demonstrate synergism with the cell wall active agent. High-level aminoglycoside resistance in enterococci is usually caused by enzymatic inactivation of the drugs, and the enzymes that destroy gentamicin also destroy tobramycin, amikacin, and kanamycin (Table 13.5). Consequently, none of these agents is used in treating serious infections caused by enterococci with high-level gentamicin resistance. If the isolate does not have concomitant high-level streptomycin resistance, however, streptomycin could be used, although some isolates have high-level resistance to gentamicin and streptomycin.

In vitro tests for the detection of high-level aminoglycoside resistance include broth, agar, or disk diffusion methods. For screening, gentamicin is tested at concentrations of 500 μ g/mL and streptomycin at 2000 μ g/mL (agar) or 1000 μ g/mL (broth). The tests are performed as described for routine dilution tests, and growth at the high concentration indicates that the isolate has high-level resistance to the agent tested. Disk diffusion tests have also been described that use special potency disks containing high amounts of gentamicin (120 μ g) or streptomycin (300 μ g).

Table 13.5 Aminoglycosides represented when high concentrations of gentamicin and streptomycin are tested to determine high-level aminoglycoside resistance in enterococci	
Agent tested	Aminoglycoside(s) represented
Gentamicin ^a	Gentamicin
	Amikacin
	Kanamycin
	Tobramycin
Streptomycin ^b	Streptomycin
^a An isolate that has high-level resistance to gentamicin also has high-level resistance to the other aminoglycosides listed; enzymes that modify gentamicin also modify the other agents.	
^b An isolate that has high-level resistance to streptomycin has high-level resistance to this agent only, unless it also has high-level resistance to gentamicin as indicated by testing high concentrations of gentamicin.	

Detection of vancomycin resistance in enterococci

The incidence of vancomycin resistance in enterococci increased sharply in the 1990s and the early part of the first decade of this century. Among clinical isolates, *E. faecium* is the most common species demonstrating vancomycin resistance followed by *E. faecalis*. The most common mechanisms are the *vanA* and *vanB* genes, but other resistance genes have been described. Isolates that are highly resistant to vancomycin can be readily detected when tested by conventional antimicrobial susceptibility test methods. Some isolates, however, may have a more subtle type of vancomycin resistance, whereby MICs or inhibition zone measurements are just slightly above or below, respectively, the breakpoints. Hence dilution tests must be viewed closely, and inhibition zones in disk diffusion testing must be examined using transmitted (rather than reflected) light; any growth within the zone should be considered significant. Vancomycin screen agar can be used to detect vancomycin resistant enterococci as well.

Low-level vancomycin resistance is intrinsic in the motile enterococcal species *Enterococcus gallinarum* and *Enterococcus casseliflavus*. These differ from true VRE for infection control purposes. In addition, vancomycin-variable enterococci have been described. These isolates initially appear susceptible to vancomycin but possess the *vanA* gene (detected by molecular methods) and can develop into vancomycin-resistant strains *in vivo*; therefore they should be reported as vancomycin resistant. *Leuconostoc*, *Pediococcus*, and *Lactobacillus* spp. demonstrate intrinsic, high-level vancomycin resistance; these bacteria must be carefully separated from the morphologically similar enterococci and streptococci.

Extended-spectrum β -lactamases

Most *K. pneumoniae*, *Klebsiella oxytoca*, and many *E. coli* isolates are resistant to ampicillin because of the production of a plasmid-mediated β -lactamase: *TEM-1* or *SHV-1*. Most isolates are susceptible to later-generation cephalosporins and aztreonam; however, spontaneous mutations occur that can result in novel **β -lactamases** that can inactivate extended-spectrum cephalosporins, penicillins, and aztreonam. These β -lactamases are known as **extended-spectrum β -lactamases** (ESBLs). The enzymes are characterized and numbered based on their relationship to the parent enzymes. There are now more than 130 TEM-derived ESBLs and more than 60 derived from SHV-1. In addition, other novel β -lactamases have been designated as CTX-M or OXA that are not a result of mutations of the TEM or SHV parent enzymes. Some of these β -lactamases give rise to subtle or difficult to detect resistance among cephalosporins, penicillins, or aztreonam. Recently these enzymes have been found in other genera and species, including *Proteus mirabilis*, *Salmonella* spp., and *Enterobacter* spp. most notably.

Strategies for laboratory detection of ESBL-producing *E. coli*, *Klebsiella* spp., and *P. mirabilis* include testing of drugs that are most likely to indicate the presence of an ESBL (indicator drugs). These include, in decreasing order of sensitivity for detection, cefpodoxime, ceftazidime, cefotaxime, ceftriaxone, and aztreonam and the use of special screening zone or MIC breakpoints to facilitate recognition of ESBL production. Indicator drugs have been selected based on the likelihood of their being readily hydrolyzed by one of the many types of ESBLs. The use of more than one antimicrobial agent increases the sensitivity of detection.

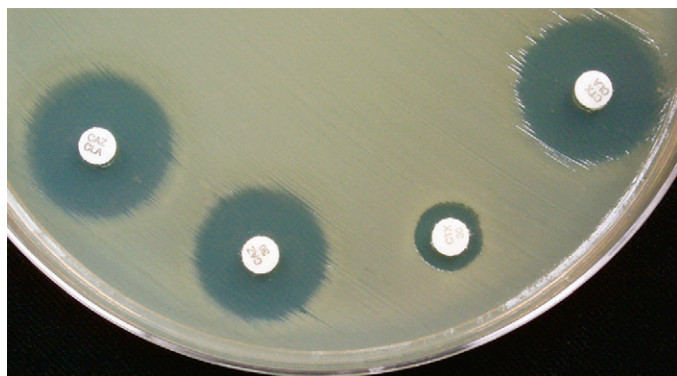


Fig. 13.16 Extended spectrum β -lactamase (ESBL) phenotypic confirmatory testing of *Klebsiella pneumoniae*. The standard disk diffusion test is performed with cefotaxime and ceftazidime, with and without clavulanic acid. The diameter of the zone around cefotaxime–clavulanic acid (4-o'clock position) is more than 5 mm larger than the zone around cefotaxime (5-o'clock position), which indicates a positive reaction as clavulanic acid restores the activity of cefotaxime. Although the zones for ceftazidime and ceftazidime–clavulanic acid are comparable, only one set of drugs must be positive to confirm an isolate as an ESBL producer.

Once an ESBL-producing isolate is presumptively detected, confirmatory testing is optional per CLSI, because the breakpoints for the third- and fourth-generation cephalosporins have been lowered. ESBL activity is inhibited by β -lactamase inhibitor agents such as clavulanic acid; therefore this property forms the basis of the confirmatory tests. If the activity of cefotaxime, ceftazidime, or both is restored when tested in combination with clavulanic acid by disk diffusion or an MIC test, the resistance is caused by ESBL production. Fig. 13.16 shows an ESBL confirmatory test performed using the disk diffusion method.

Resistance in ESBL-producing strains to various β -lactam agents is not always predicted by in vitro tests performed using standard inoculum density. For example, a confirmed ESBL-producing *K. pneumoniae* isolate may appear susceptible to cefotaxime by a disk diffusion or MIC test; however, cefotaxime is hydrolyzed at a high inoculum density and is ineffective in treating infections caused by these strains. Consequently, when an ESBL-producing isolate is identified and confirmed, it should be reported as clinically resistant to all cephalosporins, penicillins, and aztreonam, despite the in vitro test results. The carbapenems (imipenem, meropenem, ertapenem) are active against ESBL-producing strains as are the cephamycins (cefoxitin and cefotetan). ESBL-producing isolates show variable susceptibility to aminoglycosides, fluoroquinolones, and trimethoprim-sulfamethoxazole, although many isolates show multiple drug resistance to those agents.

Carbapenemases

Carbapenemase-producing Enterobacterales (CPE) are increasingly seen in the clinical setting. These organisms carry resistance genes, often on plasmids, to carbapenem antibiotics as well as penicillins, monobactams, and cephalosporins to different degrees. It is vitally important to detect CPE for effective patient treatment and infection control purposes.

Klebsiella pneumoniae carbapenemase

K. pneumoniae carbapenemases (KPCs) are the most common type of CPE in the United States. Initially identified

in *K. pneumoniae*, they have been recognized in a variety of other members of the Enterobacterales, as well as some nonfermenters, such as *Pseudomonas aeruginosa*. Organisms possessing these enzymes are often resistant to one or more of the carbapenems. If an isolate possesses an ESBL and the MIC for a carbapenem is 2 or 4 $\mu\text{g/mL}$, there is a possibility that it may produce a KPC-type or some other carbapenemase.

Molecular methods can detect carbapenemase genes, but these tests are limited in terms of required expertise, time, and sample number. A simple, objective, inexpensive phenotypic method, the carbapenem inactivation assay, was endorsed by the CLSI in 2017. In this test, a carbapenem disk is incubated in water or trypticase soy broth (TSB) containing a suspension of a suspected carbapenemase-producing organism for 4 hours. Inactivation of the disk is analyzed by placing that carbapenem disk on a lawn plate of a carbapenem-susceptible control strain, resulting in no zone of inhibition after overnight incubation. Lateral flow assays, such as the NG-Test Carba 5 (Hardy Diagnostics, Santa Maria, CA), have been developed that will detect up to 5 different carbapenemases. In vitro susceptibility testing of members of the Enterobacterales containing KPCs may indicate that the isolate is susceptible to carbapenems, but therapeutically the carbapenem antimicrobial might not be effective. Infections often are treated with colistin or polymyxin with mixed results.

β -Lactamase tests

As mentioned previously, the β -lactamases inactivate some β -lactam antibiotics. Production of β -lactamase is a significant mechanism contributing to antimicrobial resistance in organisms, such as *Staphylococcus* spp., *H. influenzae*, *N. gonorrhoeae*, *M. catarrhalis*, several *Bacteroides* spp., and occasionally *Enterococcus* spp. and *Pseudomonas* spp. β -Lactamases are the primary mode of resistance to β -lactam antibiotics. Another method discussed earlier is altered PBPs with lower affinity to the β -lactams.

Several tests can be performed in the clinical laboratory to identify β -lactamase production, and the rapidity of β -lactamase tests makes them attractive as a means for direct detection of one important resistance determinant. A positive result means that the β -lactam agents commonly used (ampicillin, amoxicillin, and penicillin) to treat infections caused by the organism would be ineffective. Some β -lactams (e.g., oxacillin), however, are resistant to β -lactamase. A negative reaction indicates that the test organisms do not produce β -lactamase, although they may be resistant to these agents through another mechanism. Resistance related to other mechanisms can be detected only with conventional dilution or disk diffusion tests. Some organisms, such as members of the order Enterobacterales and the *Pseudomonas* spp., produce a variety of different types of β -lactamases; the currently available direct β -lactamase tests cannot reliably predict resistance to the newer β -lactam agents that might be used for these organisms, so β -lactamase testing should not be performed on them.

The most accurate means to determine β -lactamase production is by detecting the *blaZ* gene with real-time PCR. The most used direct detection method incorporates the chromogenic cephalosporin nitrocefin. Cefinase disks BD BBL (Thermo Fisher Scientific, Waltham, MA) are a commercial

product consisting of filter paper disks impregnated with nitrocefin approved for use on isolated colonies of *N. gonorrhoeae*, *Staphylococcus* spp., *H. influenzae*, enterococci, and anaerobic bacteria. A disk is moistened with water or saline, and a loopful of organisms is applied directly onto the disk. A β -lactamase-producing organism will turn red within 10 minutes (or within 60 minutes for staphylococci). No color change indicates a β -lactamase-negative organism (Fig. 13.17).

Other less commonly used β -lactamase tests utilize penicillin-based acidimetric and iodometric tests that detect the production of penicilloic acid from the hydrolysis of penicillin by a β -lactamase. The acidimetric method uses citrate-buffered penicillin and phenol red as a pH indicator. When β -lactamase-producing bacteria are added to the solution, the penicilloic acid results in a drop in pH, causing a color change from red to yellow. In the iodometric method, a solution of phosphate-buffered penicillin and starch-iodine complex is used. With β -lactamase-positive organisms, penicilloic acid reduces iodine and prevents it from combining with starch. A positive reaction is colorless, and a negative reaction is purple.

Most bacteria, except staphylococci, produce β -lactamase constitutively, meaning that the same amount of enzyme is produced regardless of exposure to an inducing agent. Production of β -lactamase in staphylococci is inducible, and exposure to an inducing β -lactam agent is often required to obtain a high enough concentration of the enzyme for detection with conventional β -lactamase tests. Studies have indicated that the nitrocefin test has low sensitivity for detecting β -lactamase activity in *S. aureus*. Testing organisms that were exposed to an inducing agent can be accomplished by using growth from the periphery of a zone surrounding a β -lactam disk. Taking the inoculum directly from the zone edge of a cefoxitin disk (30 μ g) from a standard disk diffusion test can increase the sensitivity. This is particularly true for the coagulase-negative *Staphylococcus* spp.

Studies showed that the penicillin disk zone edge test is the most sensitive method, and the CLSI recommends this test for determining β -lactamase production in *S. aureus*. For the CLSI protocol, a 10-unit penicillin disk is used in a standard disk diffusion test. The breakpoint for resistance is a zone <29 mm. Isolates with a sharp zone

edge are considered positive for β -lactamase production, while those with a feathered edge are regarded as negative. Reading the results is highly subjective; zones must be examined carefully with reflected light. The European Committee on Antimicrobial Susceptibility Testing recommends using a 1-unit penicillin disk and a breakpoint of <26 mm; studies indicate it to be more sensitive in detecting β -lactamase production in staphylococci. The CLSI does not recommend the zone edge test for coagulase-negative *Staphylococcus* spp.

Etest

The Etest uses the principle of an antimicrobial density gradient in agar medium as a means of determining antimicrobial susceptibility. The Etest uses thin plastic test strips impregnated on the undersurface with an antimicrobial concentration gradient and marked on the upper surface with a concentration index or scale. The strips may be placed in a radial fashion on the surface of an agar plate that has been inoculated in a manner like that for a disk diffusion test. After overnight incubation, the test results are read by viewing the plates from the top, with the lids removed. The antimicrobial gradient that forms in the agar around the Etest strips gives rise to an elliptic inhibitory area around each strip. The MIC is determined by where the growth ellipse intersects the Etest strip (Fig. 13.18). Like the disk diffusion test, the Etest has the flexibility of drug selection and testing because selected strips are applied to the surface of test plates. The cost of Etest strips is much more than that of antimicrobial disks, however, and is the main limitation of this product. Generally, published studies have indicated that MICs determined using the Etest compare favorably (within one twofold dilution interval) with those determined by conventional broth or agar dilution methods.

The Etest can be especially useful for testing fastidious organisms such as *S. pneumoniae*, other streptococci, *H. influenzae*, and anaerobic bacteria, in part because the strips can be placed on enriched media or in a special incubation atmosphere (e.g., increased CO₂ or anaerobic) and because relatively few antimicrobial agents may need to be tested against fastidious organisms. Consequently, the relatively high cost of the Etest strips could be minimized.



Fig. 13.17 Cefinase β -lactamase disk test. Cells from several colonies of *Haemophilus influenzae* were applied to a moistened disk. This photo shows results after testing of two different isolates. Within 10 minutes, the disk on the left turned brown-red (positive), and that on the right maintained a light yellow color (negative).



Fig. 13.18 *Escherichia coli* tested with an Etest gentamicin strip. The gentamicin minimal inhibitory concentration (where the ellipse crosses the gradient) is 0.75 μ g/mL.

Automated antimicrobial susceptibility test methods

Principles of technologies used

The automated susceptibility testing instruments currently available represent a choice of several different levels of automation. One instrument interprets growth end points of broth microdilution panels only when they are placed into an automated reader device, whereas other instruments provide hands-off incubation and reading functions for microdilution trays or special cards in an incubator-reader device. The instruments that offer the highest level of automation accomplish these tasks using robotics to move the panels or cards in the instrument during the incubation-reading sequences or to add reagents to certain test wells for biochemical tests.

Current instruments use one of two optical approaches for examining the test wells of the antimicrobial-containing panels or cards. Most instruments use the principle of turbidimetric detection of bacterial growth in a broth medium in test wells by a photometer. The determination of antimicrobial susceptibility is based on the lack of development of turbidity (growth suppression) or, conversely, an indication of resistance based on an increase in turbidity in the presence of an antimicrobial agent. The second means of recognizing growth is detecting hydrolysis of a fluorogenic growth substrate incorporated in a special test medium. With this method, growth is detected by a fluorometer as emission of a fluorescent signal when a microorganism consumes fluorophore-labeled substrate in the test medium during growth.

Instruments for antimicrobial susceptibility testing can provide test results after a conventional overnight incubation period or in a period of 5 to 15 hours. Instrumentation can interpret antimicrobial susceptibility test end points sooner than manual readings because of the greater sensitivity of the instruments' optical systems in detecting subtle increases in microbial growth. All of the instruments utilize computers to provide reports and to store and retrieve data on antimicrobial susceptibility. Most of the instruments can also be used to identify bacteria and to merge and print identification and antimicrobial susceptibility results into a single report and transfer the information to a laboratory information system (LIS).

Automated systems

Reader devices for broth microdilution susceptibility tests

Commercial manufacturers of broth microdilution antimicrobial susceptibility testing panels offer a view box or other device to facilitate the manual reading of results after incubation. They offer freeze-dried antimicrobial panels with a mechanized device to simplify hydration and inoculation of freeze-dried panels. Some manufacturers provide frozen panels. The Sensititre Vizion (Thermo Fisher Scientific) offers an instrument-assisted manual reader that allows the laboratory scientist to record the results using customizable lighting options to produce an easy-to-read digital plate image.

A similar instrument is the MicroScan TouchScan (Beckman Coulter Pasadena, CA). The MicroScan AutoSCAN-4 is a semi-automated system. After trays are incubated offline, the laboratory scientist inserts a tray into the reader that interprets growth patterns in panels by turbidimetric analysis.

Automated instrument systems

Currently, four instruments are available that can generate rapid (5 to 15 hours) or overnight (16 to 24 hours) susceptibility test results.

BD Phoenix system

The BD Phoenix automated microbiology system (BD Diagnostic Systems, Sparks, MD) is marketed in Europe, Japan, Canada, and the United States. The original Phoenix system consists of an instrument with a built-in keyboard and bar-coding station that can accommodate 100 test panels simultaneously, along with a separate printer (Fig. 13.19). The specially designed test cartridges contain 136 small wells (Fig. 13.20) to test as many as 25 different antimicrobial agents, alone or in combination with wells containing biochemical substrates for simultaneous identification of common gram-positive or gram-negative bacteria (combo panels). The test panels are inoculated manually by a simple, gravity-fed transfer of inoculated medium throughout the disposable cartridge after pouring inoculated broth through an opening in the test device. After inoculation, the panels are placed into the instrument's incubator. The Phoenix system



Fig. 13.19 BD Phoenix automated microbiology system. (Courtesy BD Diagnostic Systems, Sparks, MD.)

checks the panels for growth in the test wells every 20 minutes using a redox indicator system, resembling resazurin, and turbidity. The indicator is added to the broth at the time of organism inoculation. This approach allows susceptibility determinations after approximately 6 to 8 hours of incubation. The Phoenix instrument includes a rules-based expert system (BDXpert) and can be purchased with a data management personal computer and software package called *BD EpiCenter*, which allows networking with other BD microbiology instruments and a bidirectional interface for connection with an LIS.

MicroScan WalkAway system

The first MicroScan WalkAway system (Beckman Coulter) was developed in the 1980s. The current fourth-generation system, the WalkAway *plus*, consists of a self-contained incubator-reader unit with capacity for 40 or 96, 96-well panels, LabPro information management software, a printer, and an LIS interface (Fig. 13.21). The WalkAway uses microdilution panels that are hydrated and inoculated with a hand-operated inoculator device (MicroScan Renok, Siemens Healthcare Diagnostics, Tarrytown, NY; Fig. 13.22). Panels are

then placed in the incubator module. The type of test to be performed is indicated on an instrument-readable bar-code label located on the end of each panel. The instrument incubates the panels for the appropriate period (depending on the type of panel and organism), robotically positions the trays to add reagents for biochemical tests, and moves them under the photometer to perform growth readings.

Standard panels are read turbidimetrically after 16 to 18 hours of incubation with 24-hour holds for specific bacteria (e.g., oxacillin for *Staphylococcus* and vancomycin for *Enterococcus*). The instrument offers the possibility of early, read-when-ready interpretations of the standard panels in 4.5 to 6.5 hours if an isolate is unequivocally susceptible or highly resistant. Organisms with inducible or slow to be expressed resistance are automatically extended to 16- or 18-hour incubation. MIC or breakpoint panels are available for testing of gram-positive and gram-negative bacteria as are special combination panels that allow simultaneous susceptibility and organism identification in the same panel. With the standard panels, it is possible to read the panels visually in case of instrument malfunction. Panels containing lysed horse blood (MICroStrep, Beckman Coulter) are also provided for testing

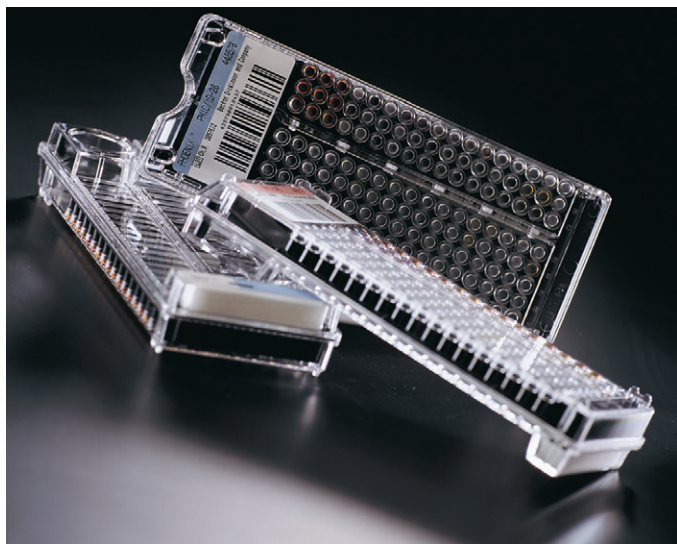


Fig. 13.20 BD Phoenix test cartridges, which contain 136 wells of antimicrobial agents or biochemical tests.



Fig. 13.22 MicroScan Renok inoculator device, which is used to reconstitute and inoculate dried antimicrobial agents or biochemical tests in the test panels. A test panel is also shown. (Courtesy Siemens Healthcare Diagnostics, Tarrytown, NY.)



Fig. 13.21 MicroScan WalkAway SI. (Courtesy Siemens Healthcare Diagnostics, Tarrytown, NY.)

streptococci that can be read manually or by an automated system. The instrument includes a rules-based expert system (LabPro Alert_{EX}) and can be purchased with a software package called *LabPro* that allows networking with other MicroScan analyzers and workstations and a bidirectional interface with an LIS.

Sensititre ARIS 2X

The Sensititre ARIS 2X (Thermo Fisher Scientific) automated incubator reader was the first system to be marketed in the United States (in 1992) that used a fluorometric detection system for detecting growth end points of common, rapidly growing bacteria. It was found that the fluorogenic substrate system could not accurately detect some resistance mechanisms in 5 hours. Therefore the system has FDA approval for only 16- to 24-hour incubation for susceptibility testing.

The Sensititre ARIS 2X (Fig. 13.23) is a fully automated, benchtop incubating and reading system. The system utilizes up to 64, 96-well trays. The AutoReader uses an internal bar-code scanner to identify each plate type and assign the appropriate incubation time; when the assigned time has elapsed, the plate is then transported to the OptiReader for fluorescence measurement, with no manual intervention. Panels for *M. tuberculosis* are also available.

VITEK systems

The VITEK systems (bioMérieux) were originally designed for use in the U.S. space exploration efforts of the 1970s as an onboard test system for spacecraft exploring other planets for life. Because of its original design intention, it was highly automated and relatively compact. Small plastic reagent cards, similar in size to a credit card, contain microliter quantities of various concentrations of antimicrobial agents in 64 wells for susceptibility and identification testing. The VITEK 2 (Fig. 13.24) can accommodate up to 60 cards, while the VITEK 2XL holds up to 120 cards. The newest VITEK 2 Compact for small laboratories is available in three sizes: 15, 30, or 60 cards. The susceptibility cards allow quantitative MIC results accompanied by susceptible, intermediate, or resistant results interpretations for most rapidly growing, gram-positive and gram-negative aerobic bacteria in 4 to 18 hours. The VITEK 2 can test *S. pneumoniae* in addition to

the nonfastidious, aerobic gram-positive and gram-negative bacteria. The VITEK 2 offers one of the highest levels of automation currently available in microbiology instrumentation.

VITEK hardware consists of a filling module for inoculation of the cards, an incubator-reader module that incorporates a carousel to hold the test cards, a robotic system to manipulate the cards, a photometer for turbidimetric measurement of growth every 15 minutes, and a computer module with a video display terminal and printer for viewing and printing results. VITEK also offers a data management system (Observe) for storing and retrieving test data for a variety of epidemiologic reports.

The VITEK 2 Compact is a less automated version of the VITEK 2 that uses the same cards but without the smart carrier and programmed cassette for specimen handling automation available in the VITEK 2. It includes a compact, self-contained vacuum chamber and card sealer. The instrument is a less expensive option for laboratories that do not require the level of automation provided by the smart carrier of the VITEK 2. All VITEK instruments use kinetic measurements of growth in the presence of antimicrobial agents to provide analysis of growth curves, leading to computer algorithm-derived MICs. All instruments include a rules-based expert system, Advanced Expert System, to assist in controlling common technical errors and facilitate detection of unusual resistance mechanisms before results are reported.

Quality control of antimicrobial susceptibility tests

QC of antimicrobial susceptibility tests involves testing reference strains with defined antimicrobial susceptibility or resistance to the drugs tested. It is important to use QC strains that represent the types of patient isolates tested in the respective laboratory. Also, the QC strains should represent various degrees of susceptibility or resistance. Ideal QC strains for MIC tests have what are termed *on-scale MIC end points* for the drugs tested. An on-scale end point falls within the range of concentrations tested compared with off-scale end points, in which the MIC is less than the lowest or greater than the highest concentration tested.

The CLSI has identified American Type Culture Collection (ATCC) strains useful for QC testing (Table 13.6). The CLSI documents also include tables that define acceptable results



Fig. 13.23 Sensititre ARIS 2X. (Courtesy TREK Diagnostic Systems, Cleveland, OH.)



Fig. 13.24 VITEK 2 System. (Courtesy bioMérieux Vitek, Durham, NC.)

Table 13.6 Commonly used strains for quality control of routine antimicrobial susceptibility tests

Test	QC strains used	Comments
Antimicrobial susceptibility of gram-positive organisms	<i>Staphylococcus aureus</i> ATCC 25923 <i>S. aureus</i> ATCC 29213	β -Lactamase negative for disk diffusion tests β -Lactamase positive for MIC tests
Oxacillin salt agar screen for <i>S. aureus</i>	<i>S. aureus</i> ATCC 43300	Oxacillin-resistant
Vancomycin BHI screen, synergy screen for enterococci and <i>S. aureus</i>	<i>Enterococcus faecalis</i> ATCC 29212 <i>E. faecalis</i> ATCC 51299	Susceptible to vancomycin and to high levels of gentamicin and streptomycin (synergy screen tests negative) Resistant to vancomycin and to high levels of gentamicin and streptomycin (synergy screen tests positive)
Antimicrobial susceptibility of gram-negative organisms	<i>Escherichia coli</i> ATCC 25922 <i>Pseudomonas aeruginosa</i> ATCC 27853 <i>E. coli</i> ATCC 35218	β -Lactamase positive for testing β -Lactam- β -lactamase inhibitor combination agents only
ESBL test	<i>Klebsiella pneumoniae</i> ATCC 700603	ESBL screen test and ESBL confirmatory test positive
Antimicrobial susceptibility of <i>Haemophilus</i> spp.	<i>Haemophilus influenzae</i> ATCC 49247 <i>H. influenzae</i> ATCC 49766 <i>H. influenzae</i> ATCC 10211	Ampicillin-resistant, non- β -lactamase producing Ampicillin-susceptible Used by media manufacturers to assess growth-supporting capabilities of the medium
Antimicrobial susceptibility of <i>Neisseria gonorrhoeae</i>	<i>N. gonorrhoeae</i> ATCC 49226	β -Lactamase negative
Antimicrobial susceptibility of <i>S. pneumoniae</i> and other streptococci	<i>Streptococcus pneumoniae</i> ATCC 49619	Penicillin-intermediate
Antimicrobial susceptibility of anaerobes	<i>Bacteroides fragilis</i> ATCC 25285 <i>Bacteroides thetaotaomicron</i> ATCC 29741 <i>Eubacterium lentum</i> ATCC 43055	
Assessment of acceptability of medium (low thymine and thymidine content) for testing sulfonamides, trimethoprim, and trimethoprim-sulfamethoxazole	<i>E. faecalis</i> ATCC 29212	

ATCC, American Type Culture Collection; BHI, brain-heart infusion; ESBL, extended-spectrum β -lactamase; MIC, minimal inhibitory concentration; QC, quality control.

(zone measurements for disk diffusion tests) for these strains; examples are shown in Table 13.7. The procedure followed in testing QC reference strains must be identical to that used for testing patient isolates. If results with QC strains do not fall within the defined acceptable limits, corrective action must be taken to determine the reason for the out-of-control observation before reporting any patient results. QC testing is recommended each day that patient tests are performed; however, the frequency of QC testing can be reduced to weekly if a laboratory can demonstrate acceptable performance with the QC strains. This consists of obtaining results within acceptable limits for each antimicrobial agent–QC strain combination for 20 or 30 consecutive test days. QC procedures must be performed when new lots of materials are put into use, and test materials must never be used beyond their stated expiration dates.

Supplemental QC strains may be periodically tested to validate acceptable performance of specific antimicrobial agent–organism combinations that may be only modestly controlled with routine reference strains. For example, a

MRSA strain could be included to ensure that the test system can detect heteroresistant strains. Similarly, ampicillin-resistant *Enterobacter cloacae* might be included to ensure that the system can detect ampicillin resistance in gram-negative bacteria. Supplemental QC strains are sometimes used for troubleshooting specific problems or training new employees. Another component of a QC program involves the inclusion of mechanisms to ensure that those performing the testing are proficient in their tasks. Self-assessment checklists and supervisory reviews of reported results are examples of such mechanisms. Satisfactory performance on proficiency survey specimens and the use of relevant testing strategies are also QC parameters.

The most widely used supplemental QC measure is antibiograms to verify results generated on patient isolates. An **antibiogram** is the antimicrobial susceptibility profile of a bacterial isolate to a battery of antimicrobial agents. Some species have typical antibiograms, which can be used to verify the identification and susceptibility results on the isolate (Table 13.8). For example,

Table 13.7 Acceptable limits for quality control strains used to monitor accuracy of disk diffusion testing of nonfastidious organisms^a

Antimicrobial agent	Disk content (µg)	<i>Escherichia coli</i> ATCC 25922 (mm)	<i>Staphylococcus aureus</i> ATCC 25923 (mm)	<i>Pseudomonas aeruginosa</i> ATCC 27853 (mm)	<i>Escherichia coli</i> ATCC 35218 (mm)
Ampicillin	10	15–22	27–35	—	6
Amoxicillin–clavulanic acid	20–10	18–24	28–36	—	17–22
Cefazolin	30	21–27	29–35	—	—
Gentamicin	10	19–26	19–27	17–23	—

^aUsing Mueller-Hinton medium without blood or other supplements.

ATCC, American Type Culture Collection.

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Table 13.8 Typical antibiograms for several gram-negative species^a

Antimicrobial agent	<i>Escherichia coli</i>	<i>Enterobacter cloacae</i>	<i>Proteus mirabilis</i>	<i>Pseudomonas aeruginosa</i>	<i>Stenotrophomonas maltophilia</i>
Amikacin	S	S	S	S	R
Ampicillin	S	R	S	R	R
Ampicillin-sulbactam	S	R	S	R	R
Cefazolin	S	R	S	R	R
Cefoxitin	S	S-R	S	R	R
Cefotaxime	S	S-R	S	S-R	R
Ceftazidime	S	S	S	S	S-R
Ciprofloxacin	S	S	S	S	R
Gentamicin	S	S	S	S	R
Imipenem	S	S	S	S	R
Piperacillin	S	S	S	S	R
Nitrofurantoin	S	S	R	R	R
Tobramycin	S	S	S	S	R
Trimethoprim-sulfamethoxazole	S	S	S	R	S

^aResults indicated represent the typical response found in most clinical isolates; however, these can vary significantly.

R, Resistant; S, susceptible; S-R, variable result.

P. aeruginosa is typically resistant to ampicillin, cefazolin and other first- and second-generation cephalosporins, and trimethoprim-sulfamethoxazole; however, it is often susceptible to gentamicin and other aminoglycosides, extended-spectrum penicillins (e.g., piperacillin), and ciprofloxacin. In contrast, *E. coli* is generally susceptible to all of the antimicrobial agents mentioned. Table 13.9 shows several atypical antibiograms suggesting that the result highlighted is erroneous. The CLSI provides a listing of results that should be verified because they (1) have never been encountered, (2) are uncommon, or (3) represent results that could easily occur because of technical errors and might have significant clinical consequences. One list includes susceptibility test results for certain genera or species that should be verified by all laboratories, and another includes results that need not be verified in facilities in which the results are common. Some of these listings are shown in Table 13.10.

When atypical antibiograms are seen, the results must be verified. Verification procedures include:

- Reexamination of the disk diffusion plate, MIC tray, and other components to ensure that results were properly interpreted and that the materials were not overtly defective (e.g., empty well in the tray)
- Checking earlier reports to see whether the particular patient previously had an isolate with an atypical antibiogram that was verified
- Repeating the test, if necessary. Sometimes it is necessary to repeat the identification and antimicrobial susceptibility tests to verify the atypical results; sometimes testing with an alternative method is useful.

With emerging resistance and healthcare-associated transmission of resistant organisms, there is now more variability in susceptibility profiles among clinical isolates than previously seen. In contrast with what was seen a decade ago, it would not be uncommon now for a facility to see a high percentage of isolates resistant to multiple antimicrobial agents.

Cumulative antibiograms are generated by the analysis of individual susceptibility results obtained on isolates from a

Table 13.9 “Problem” antibiograms suggestive of technical errors or patient issue

Antimicrobial agent	<i>Escherichia coli</i> ^a	<i>Enterobacter cloacae</i> ^b	<i>Pseudomonas aeruginosa</i> ^c	<i>Stenotrophomonas maltophilia</i> ^d
Amikacin	R	S	S	R
Ampicillin	S	R	S	R
Cefazolin	S	S	R	R
Cefoxitin	S	R	R	R
Cefotaxime	S	R	S-R	R
Gentamicin	S	S	S	R
Tobramycin	S	S	S	R
Trimethoprim-sulfamethoxazole	S	S	R	R

^aIt is very unusual for an isolate to be resistant to amikacin and susceptible to gentamicin and tobramycin because amikacin is typically the most active of these three aminoglycosides.

^bThird-generation cephalosporins (e.g., cefotaxime) are usually more active than second-generation cephalosporins (e.g., cefoxitin), which in turn are more active than first-generation cephalosporins (e.g., cefazolin) against the Enterobacterales. In addition, *E. cloacae* is typically resistant to cefazolin. Consequently, this antibiogram is unusual.

^cAmpicillin does not produce activity against *P. aeruginosa*, and this antibiogram is unusual.

^d*S. maltophilia* is usually susceptible to trimethoprim-sulfamethoxazole, which is the drug of choice for infections caused by this very resistant species. This antibiogram is unusual.

R, Resistant; S, susceptible; S-R, variable result.

Table 13.10 Suggestions for verification of antimicrobial susceptibility test results and confirmation of organism identification

Organism or group	Category I: verify at all laboratories	Category II: verify; institution specific
<i>Klebsiella</i> spp.	Ampicillin, S Automatically reported R regardless of result in some laboratories	ESBL confirmed positive Some instruments can reliably do this testing (i.e., Phoenix)
<i>Streptococcus pneumoniae</i>	Fluoroquinolone, R Linezolid, NS Vancomycin, NS	Penicillin, R Third-generation cephalosporin, R

ESBL, Extended-spectrum β -lactamase; NS, not susceptible; R, resistant; S, susceptible.

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particular institution in a defined period; this represents the percentage of isolates of a given species susceptible to the antimicrobial agents commonly tested against that species (e.g., the percentage of *E. coli* isolates that are susceptible to ampicillin). Cumulative antibiograms are generally compiled annually to guide physicians in empiric therapy decisions. If a physician were treating a patient with a suspected infection caused by *P. aeruginosa* and the culture and susceptibility test results were not yet available, the physician could review the cumulative antibiogram to see the percentage of *P. aeruginosa* isolates in that facility susceptible to various antipseudomonal agents. This information could assist the physician in designing the empiric therapy regimen pending completion of culture and susceptibility testing. Cumulative antibiogram data also might be used for infection control purposes. An increase in the incidence of MRSA from 25% in the first quarter of the year to 60% in the second quarter might suggest a problem with healthcare-associated transmission of MRSA

among the patients in that facility. An investigation by infection control personnel into the reason behind such observations would be warranted.

Selecting an antimicrobial susceptibility test method

Clinical microbiology laboratories can choose from several manual or instrument-based methods to perform their routine antimicrobial susceptibility testing—the disk diffusion (or Kirby-Bauer) test, broth microdilution (with or without use of an instrument for panel readings), and rapid automated instrument methods. The Etest also can be useful for certain fastidious or obligate anaerobic bacteria.

The disk diffusion test provides the greatest flexibility and cost-effectiveness. A problem mentioned frequently with commercial microdilution or automated systems is the inflexibility of the standard antimicrobial agent batteries or test panels. With the current availability of more than 50 antimicrobial agents in the United States and the diversity among antimicrobial agent formularies in different hospitals, it is impossible for manufacturers to provide standard test panels that fit every hospital's needs. Thus the inherent flexibility of the disk diffusion test allows a laboratory to test any of 12 antimicrobial agents considered appropriate on a 150-mm Mueller-Hinton agar plate.

Other assets of the disk diffusion procedure are that it is one of the longest practiced standardized methods, and its performance is continually updated by the CLSI. The interpretive category results—nonsusceptible, susceptible, intermediate, resistant—of the disk diffusion test should be readily understandable by all physicians, which is frequently not the case with MIC results. Therefore it is useful to provide the interpretive category results along with the MIC.

As noted, commercial microdilution susceptibility test products are widespread in U.S. clinical laboratories.

Advantages of this method include its quantitative nature (an MIC rather than a category result), ability to determine MICs with some organisms for which the disk test may not be standardized, and the availability of automated panel readers. In addition, the computerized data management systems that accompany some of the instruments can be helpful to some laboratories for the storage and calculation of cumulative antibiograms and other susceptibility statistics.

A laboratory can perform automated antimicrobial susceptibility testing to reduce the amount of labor required for susceptibility tests and to generate test results more rapidly than can be accomplished by manual methods. Providing antimicrobial susceptibility tests a day or even hours sooner is a great advantage in patient care. Studies have documented a decrease in patient morbidity or mortality, as well as demonstrated substantial cost savings. If physicians are aware of critical susceptibility data on patients' isolates, an opportunity exists for improving the quality of care, quicker discharge of patients, or removal of patients from isolation or unnecessary and expensive empiric treatment (i.e., vancomycin), thus realizing cost savings.

Some time savings can be achieved with automated susceptibility instruments by using combination panels that allow antimicrobial susceptibility testing and organism identification on the same panel. In addition, some instruments provide mechanized or simplified panel inoculation and reading to achieve labor savings. The magnitude of the labor savings realized in clinical chemistry or hematology through automation has yet to be fully achieved in clinical microbiology. If a serious mechanical failure occurs, only tests from instruments that use conventional microdilution trays normally interpreted after overnight incubation can be completed by manual incubation and interpretation. The results obtained with instrument methods that incorporate rapid test interpretation or use fluorogenic substrate analysis cannot be manually interpreted. Therefore laboratories must have a backup testing procedure, such as disk diffusion or manual overnight broth microdilution testing, to avoid delays in generating patients' results.

A previous shortcoming of rapid susceptibility testing methods was the inability to detect certain inducible or otherwise subtle antimicrobial resistance mechanisms. Manufacturers have made significant strides toward solving these problems. In some cases, this has meant reformulating the test devices or improving the software used to interpret the susceptibility testing results, which can be aided in part by computer expert systems that detect common technical errors and explain some newer resistance mechanisms. Nevertheless, it is important for microbiologists to scrutinize carefully all susceptibility results before issuing final patient reports.

Rapid susceptibility determination

The successful treatment of infectious diseases requires not only the identification of the causative bacterium, but also determination of the bacterium's susceptibility to antimicrobials. Over the past several years, the rapid identification of bacteria has been more successful than the rapid determination of the bacterium's antimicrobial susceptibility. However, the detection of drug-resistant genes has demonstrated promise to increase the speed of determining a bacterium's resistance to antimicrobials. These rapid tests allow the treatment

of bacterial infections much sooner. Molecular methods for the detection of antimicrobial resistance genes are limited to those organism-antimicrobial combinations in which only a few genes are associated with the resistance, and the resistance has a high degree of clinical significance.

Currently, about eight systems have received FDA approval for nucleic acid amplification testing. They are based on two methodologies—PCR and solid-phase microarrays—and can be performed directly on clinical specimens or culture isolates. For example, the Verigene BC-GP (Luminex Corporation, Austin, TX) can identify several species of gram-positive bacteria and detect *mecA* resistance gene in *S. aureus* and *Staphylococcus epidermidis* and *vanA/B* genes in *E. faecalis* and *E. faecium* in a few hours directly from positive blood culture bottles. This assay is based on hybridization of DNA target sequences to nanoparticles and is classified as moderate complexity by CLIA. The BioFire FilmArray (bioMérieux) and GeneXpert (Cepheid, Sunnyvale, CA) are PCR-based instruments also classified as moderate complexity. Other systems are classified as high complexity. ESBL production by gram-negative bacteria is one of the greatest threats to antimicrobial therapy today. Several commercial assays for the molecular detection of ESBL exist. Some, like the FilmArray, have a single target (KPC), while others detect a variety of β -lactamases (e.g., Verigene). Probes directed toward genes responsible for other resistance mechanisms (e.g., aminoglycoside-modifying enzymes and tetracycline resistance factors) have also been described.

Special antimicrobial susceptibility tests

Clinical microbiology laboratories that perform bacterial cultures generally perform antimicrobial susceptibility tests by a disk diffusion or MIC method. These tests assess the ability of antimicrobial agents to inhibit the growth of bacteria and are sufficient for guiding antimicrobial therapy in most situations. In select cases, a physician may wish to determine how well specific antimicrobial agents kill bacteria or how the activity of a combination of antimicrobial agents compares with that of a single agent. Unlike disk diffusion and MIC tests, however, tests for bactericidal activity (e.g., MBC tests and **serum bactericidal tests** [SBTs]) and for antimicrobial synergism are not highly standardized. When patients are prescribed antimicrobial agents that have a low toxic-to-therapeutic ratio, it may be necessary to monitor the concentrations of drug in the patient's serum to avoid excessive concentrations that can be harmful. Various methods are used for these assays. Some antimicrobial susceptibility tests are generally performed only in specialized laboratories and are used in only a few defined clinical settings or for research purposes (Table 13.11).

Case in point

Viridans group *Streptococcus* was isolated from all six blood culture bottles submitted from a patient with suspected bacterial endocarditis. The isolate was susceptible to penicillin (MIC $\leq 0.03 \mu\text{g/mL}$), but the physician was concerned about the bactericidal activity of penicillin alone. The laboratory performed MBC testing and reported an MBC of $32 \mu\text{g/mL}$. The physician considered this

information in conjunction with clinical observations of the patient and documentation in the literature of previous experiences with patients with similar conditions. This patient continued with a therapeutic regimen of penicillin and gentamicin.

Issues to consider

After reading the patient’s case history, consider:

- Circumstances when it might be appropriate to do more than a disk diffusion or MIC test on a clinical isolate
- Factors that might contribute to the MBC being five twofold dilutions greater than the MIC
- Rationale for adding a second agent (gentamicin) to treat the infection

Minimum bactericidal concentration test

MIC tests identify the amount of antimicrobial agent required to inhibit the growth or multiplication of a bacterial isolate. If a concentration of antimicrobial agent that is the same as or preferably exceeds the MIC is attained at the infection site for an appropriate length of time, the drug generally inhibits multiplication of the bacteria such that the patient’s immune defense mechanisms can successfully eliminate the infecting bacteria. The immune defense mechanisms (e.g., phagocytic cells, antibody) work together with antimicrobial agents to eradicate infecting bacteria. For this reason, inhibitory concentrations of the drug at the infection site are generally sufficient for treating most infections.

In immunosuppressed patients and patients with serious infections such as endocarditis, meningitis, and osteomyelitis, immune defense mechanisms are suboptimal. Inhibitory concentrations of the drug may not be sufficient, and obtaining bactericidal concentrations of antimicrobial agents at the

infection site is important for achieving a cure. For many types of infections, the bactericidal capacity of a specific antimicrobial regimen can be predicted based on previous experience. For example, most β -lactam antimicrobial agents are bactericidal for *E. coli*, provided their MIC is in the susceptible range. On the other hand, the bactericidal activity of β -lactams and other cell wall-active agents (e.g., vancomycin) against *S. aureus* is less predictable. In circumstances in which a serious *S. aureus* infection occurs in a patient with poor immune defense mechanisms, an in vitro determination of the amount of antimicrobial agent required to kill the isolate may be helpful. The MBC test is used for this purpose. The MBC is defined as the lowest concentration of antimicrobial agent that kills 99.9% of the test bacteria. The CLSI has described procedures for assessing bactericidal activity; however, unlike disk diffusion and MIC tests, a standardized MBC test has not been in use for a sustained period. In the past, numerous procedural variations compromised the use of the test results.

The MBC test is performed in conjunction with a broth macrodilution or broth microdilution MIC test. The antimicrobial agent concentrations that show inhibition (equal to or greater than the MIC) may or may not have killed the bacteria in the test inoculum (Fig. 13.25). After the MIC determination, a 0.01-mL aliquot from each clear tube or well is subcultured onto an agar medium to determine the MBC. The numbers of colonies that grow on subculture are compared with the actual number of organisms inoculated into the MIC test tubes or wells to determine the extent of bactericidal activity at each antimicrobial concentration. If the numbers of colonies on a subculture plate total less than 0.1% of the initial inoculum (indicating 99.9% or more killing), by definition, a bactericidal effect has been achieved.

The number of bacteria in each tube (or well) immediately after inoculation of the MIC test tube or well is approximately

Table 13.11 Special antimicrobial susceptibility tests	
Test	Purpose
Antimicrobial concentration test (assay)	Measure of amount of antimicrobial agent in serum or body fluid
Minimum bactericidal concentration test	Measure of lowest concentration of antimicrobial agent that kills 99.9% of a bacterial isolate population
Serum bactericidal test	Measure of highest dilution or titer of a patient’s serum that is inhibitory to the patient’s own infecting bacterium and highest dilution or titer that is bactericidal
Synergy test	Measure of susceptibility of a bacterial isolate to a combination of two or more antimicrobial agents
Time-kill assay	Measure of rate of killing of bacteria by an antimicrobial agent (as determined by examining the number of viable bacteria remaining at various intervals after exposure to the agent)

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Fig. 13.25 Broth macrodilution test showing vancomycin and *Staphylococcus aureus*. The minimal inhibitory concentration (MIC) is 1.0 $\mu\text{g/mL}$. The purity plate shows a pure culture. The colony count plate shows 53 colonies, which means that 5.3×10^5 colony-forming units (CFU) per milliliter were in the test tubes immediately after inoculation of the MIC test tubes. For the colony count plate, immediately after inoculation, the growth control tube was diluted 1:1000, and 0.1 mL was plated. Now 0.01 mL will be plated from each clear tube (tubes containing 1.0–128 $\mu\text{g/mL}$ vancomycin) for the minimum bactericidal concentration determination. Because it was shown that the actual colony count in the MIC test was 5.3×10^5 CFU/mL, growth of five or fewer colonies would indicate a 3 $\log_{10}$ decrease, or 99.9% killing. By definition, the concentration of drug in the respective tube would be considered bactericidal.

5×10^5 CFU/mL. For the MBC test, however, an actual colony count must be performed on the test inoculum when the MIC test tube or well is inoculated. A small aliquot from the growth control tube or well is diluted in saline or broth to obtain a countable number of colonies; the final dilution is plated on an agar medium. Generally, a 1:1000 dilution is performed (0.01 mL from the growth control tube is diluted in 10 mL), and 0.1 mL is spread over the surface of an agar plate, resulting in an overall dilution of 1:10,000. After overnight incubation, the number of colonies that grew on the colony count plate are counted. Because this count represents a 1:10,000 dilution, the count is multiplied by 10^4 to determine the number of bacteria in the original growth control and the antimicrobial agent solutions. The number of colonies representing a 99.9% reduction in the original inoculum is determined. This is the MBC end point.

In the example shown in Fig. 13.25, the actual count on the colony count plate is 53; therefore the number of bacteria in each tube of the MIC test immediately after inoculation was 5.3×10^5 CFU/mL (53 times the dilution factor 10^4). A 99.9% killing (or 0.1% survival) would be accomplished if five or fewer colonies grow on subculture of each clear well or tube after reading of the MIC test (Fig. 13.26). In this example, subcultures from all tubes containing 2.0 µg/mL vancomycin or more grew fewer than five colonies. Consequently, the MBC is 2.0 µg/mL. The 99.9% end point (or 3 log₁₀ reduction in growth of the original inoculum) is an arbitrary value, with 95% confidence limits, although its clinical relevance has not been rigorously confirmed. Measurement of MBC is thus a rough prediction of bacterial elimination and can be used for research purposes to evaluate new antimicrobial drugs.

Case check 13.4

The MIC of an antimicrobial agent determines the concentration required to inhibit the growth of a bacterium. This inhibition can be bacteriostatic or bactericidal. The concentration that must be achieved in certain clinical situations (e.g., endocarditis, meningitis) must be bactericidal to affect a successful outcome for the patient. In this case, an MBC test could improve patient outcomes.

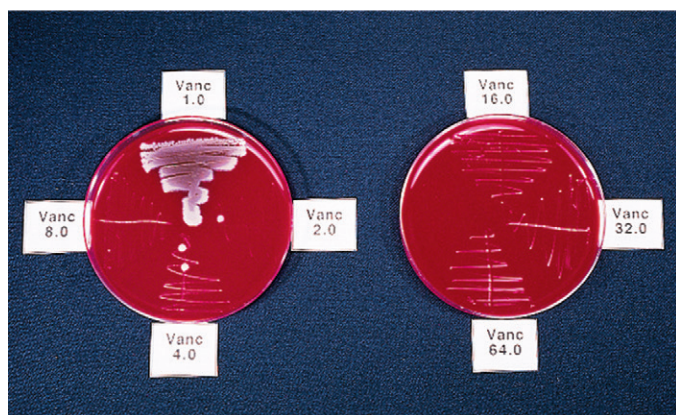


Fig. 13.26 Subculture plates from the minimal inhibitory concentration test of vancomycin and *Staphylococcus aureus* shown in Fig. 13.25. Subcultures from tubes containing 8.0 to 128 µg/mL vancomycin show no growth. Subcultures from the tubes containing 2.0 and 4.0 µg/mL show one and two colonies, respectively, indicating a greater than 99.9% killing. More than five colonies have grown from the 1.0 µg/mL tube, so the minimum bactericidal concentration is 2.0 µg/mL.

Controlling test variables

MBC tests are subject to more technical pitfalls than MIC tests, and several variables must be rigidly controlled during MBC testing. The first involves inoculum. Because many antimicrobial agents exert a bactericidal effect only on growing cells, bacteria in the midlogarithmic phase of growth must be used as the inoculum to prevent falsely elevated MBCs. The inoculum preparation methods described for MIC tests that use stationary phase growth are unacceptable for MBC tests.

Second, during inoculation for MIC tests, care must be taken to ensure that all bacteria in the test inoculum are deposited directly into the antimicrobial solution. If this is not done, bacteria may stick to the wall of the tube or well above the meniscus of the antimicrobial solution and can remain viable during incubation of the MIC portion of the test. These cells (which have not been exposed to antimicrobial agent) might then be inadvertently transferred during the subculture step, ultimately resulting in falsely elevated MBCs.

Third, the volume subcultured after reading of the MIC test must be large enough to contain sufficient inoculum but small enough to prevent carryover of large amounts of antimicrobial agent that can continue to exert an antibacterial effect. Usually, 10 µL (0.01 mL) is recommended.

Interpretation concerns

Several interpretive problems, most common with β-lactam agents, have been associated with MBC tests, and they relate to technical or biological issues. Sometimes more colonies are growing on subcultures at higher drug concentrations than at lower concentrations. This decreased bactericidal activity at higher concentrations is referred to as a *paradoxical (Eagle) effect*. Sometimes small numbers, but slightly greater than 0.1% of the test inoculum, of bacteria grow on several subculture plates. This may occur if some bacteria are metabolically inactive or dormant (**persister cells**) at the times of testing. However, when the persisting colonies are retested, their MICs are comparable with those originally obtained. Finally, tolerance to the intrinsic bactericidal effect of an antimicrobial agent is demonstrated when the number of colonies growing on subculture plates exceeds the 0.1% cutoff for several successive drug concentrations above the MIC. **Tolerance** is generally defined as an MBC/MIC ratio of 32 or higher. Tolerance has been associated with a defect in bacterial cellular autolytic enzymes.

Time-kill assays

Bactericidal activity of antimicrobial agents also can be assessed by performance of in vitro **time-kill assays**. These assays examine the dynamics of synergism or antagonism of antimicrobial agents in combination, by determining the number of viable bacteria that remain over time after exposure to each individual drug and drug combinations. Because they are labor intensive, these assays are rarely performed in the clinical microbiology laboratory. They are performed rarely in the case of patients with meningitis or endocarditis with treatment failure and in research to study novel antimicrobial drugs. CLSI does not have a standardized procedure for these assays.

Case check 13.5

In some cases, a combination of antimicrobial agents (combination therapy) is required to effect killing of the bacterium to result in treatment success. An example is the use of a β -lactam drug and an aminoglycoside to treat serious enterococcal infections. A synergy test can be performed in the laboratory to confirm that this therapy would be effective.

Synergy tests

Some types of infections require therapy with a combination of two or more antimicrobial agents. Enterococcal endocarditis, for example, requires use of a penicillin (or vancomycin) and an aminoglycoside for reliable killing of the organism. A broad-spectrum cephalosporin and aminoglycoside are often prescribed for gram-negative sepsis in neutropenic patients. The goals of combination therapy are to obtain broad-spectrum coverage, enhance antibacterial activity through synergistic interactions, and minimize drug resistance development. For most infections requiring combination therapy, single-agent MIC results and previous experience in treating similar types of infections are sufficient to guide the proper selection of an antimicrobial agent.

Synergy tests are not routinely performed in the clinical laboratory but may be useful when a patient is not responding to what would appear to be an adequate regimen, unusual organisms or resistance properties are encountered, or host factors preclude the use of certain agents. Synergy testing for multidrug-resistant gram-negative bacilli and extremely drug-resistant mycobacteria is currently under study.

In vitro synergy tests may be performed using a broth microdilution checkerboard assay or broth macrodilution method, as described by CLSI. The checkerboard assay is a type of two-dimensional test. All steps are performed as for single agents, but two agents are tested in each well or tube. Checkerboard synergy tests are usually performed in broth microdilution MIC trays. A wide variety of combinations of concentrations are tested by dispensing drugs in a two-dimensional checkerboard format, and each drug tested in the combination is also tested alone. A combination is said to show **synergism** if its antibacterial activity is significantly greater than that of a single agent; that is, when the MIC for each drug in the combination is less than or equal to 25% of the single-agent MIC. Conversely, **antagonism** is defined as the activity of the combination less than (and MICs are greater than) that of the single agents. For **indifference**, the activity of the combination is equal to that of the single agents (Fig. 13.27).

Case check 13.6

Determining that the concentration of an antimicrobial agent in the blood (serum, plasma) is sufficient to kill the bacterium causing the infection is sometimes needed to confirm that the dosing of the agent is therapeutic.

Serum bactericidal test

In the late 1940s, Schlichter and MacLean described a test that measured the effectiveness with which penicillin in serum killed bacteria associated with subacute bacterial endocarditis (SBE). This test was subsequently referred to as the serum bactericidal test (SBT). There is no single standardized SBT method, though the CLSI has described methods in an approved guideline, including broth macrodilution and broth microdilution methods.

The SBT is like the MIC-MBC test in that the serum inhibitory test (SIT) and SBT parameters are evaluated. The patient's serum during drug therapy and the bacterial isolate responsible for the patient's infection are required. Serial two-fold dilutions of the patient's serum are prepared, and then a standardized inoculum of the patient's bacterial isolate is added to each dilution. After overnight incubation, the tubes or wells are examined to determine the greatest dilution of the patient's serum that inhibits the bacteria (SIT). Subsequently, as with the MBC test, the contents of all tubes or wells showing inhibition are subcultured on an agar medium to determine the highest dilution that kills >99.9% of the bacteria (SBT). The SBT result relates to the amount of antimicrobial agent and other antibacterial factors (e.g., antibody, complement) present in the patient's serum.

Timing the collection of serum is critical, and generally trough (taken just before drug is administered) and peak (taken soon after dose is administered) titers are tested. A trough bactericidal titer of 1:32 or greater and a peak bactericidal titer of 1:64 or greater has been historically correlated with bacteriologic cure in patients with endocarditis. The described methods are controversial, and currently there are few situations in which this test is of clinical value. Current drugs used for the treatment of SBE generally achieve bactericidal levels when given for therapy, and it is very difficult to achieve and monitor serum levels of the drug in the patient accurately.

Measurement of antimicrobial agents in serum and body fluids

The amount of antimicrobial agent in serum or other body fluid can be measured by a variety of antimicrobial assay procedures. Antimicrobial assays are performed for antimicrobial agents in which the therapeutic concentration is close to the toxic concentration. Assay results often lead to modification of subsequent doses to prevent accumulation of excessive drug concentrations that might be harmful to the patient. A patient's renal status and hepatic status greatly influence in vivo levels of some antimicrobial agents. The antimicrobial agents with the greatest toxic risks and those most commonly monitored are the aminoglycosides, vancomycin, and chloramphenicol. For evaluation of antimicrobial levels, trough and peak samples should be assayed as for the SBT.

Biological assays

Antimicrobial assays were initially performed by a biological assay (bioassay) method; bioassays are still sometimes used

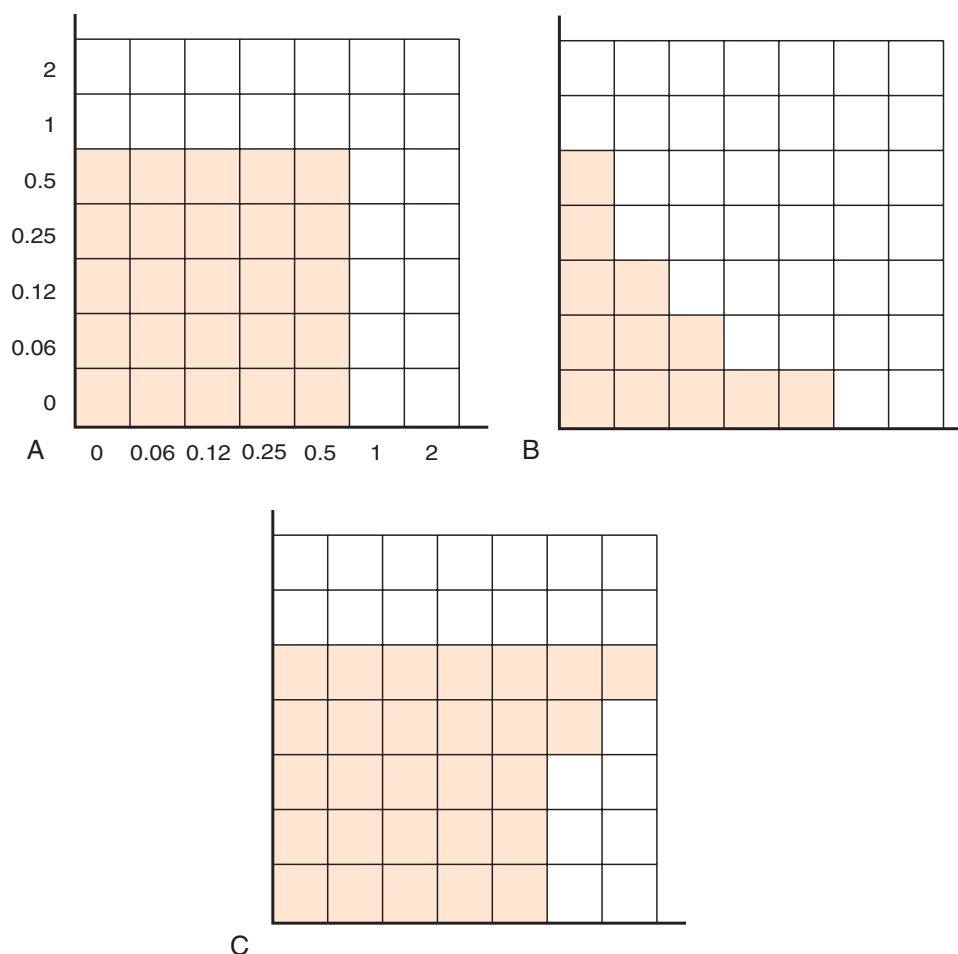


Fig. 13.27 Assessment of antimicrobial combinations with the checkerboard method. All three diagrams depict the results of testing combinations of two drugs (diluted in geometric twofold increments along the x- and y-axes, drug A along the x-axis and drug B along the y-axis). Shading indicates visible growth, and concentrations are expressed as multiples of the minimal inhibitory concentration. **A**, Indifference. **B**, Synergism. **C**, Antagonism. (Modified from Pillai, S. K., et al. (2005). *Antimicrobial combinations*. In V. Lorian (Ed.), *Antibiotics in laboratory medicine: making a difference* (5th ed.). Philadelphia: Lippincott Williams & Wilkins.)

today when the focus is on the amount of biologically active drug present, rather than the amount of “chemical” present. The bioassays use a specific strain of bacteria (indicator organism) that is susceptible to the drug to be assayed. The test can be performed in broth or on agar. The antibacterial activity of the patient’s specimen against the bacterium is compared with that of solutions containing defined concentrations of the antimicrobial being assayed to determine the concentration in the patient’s specimen using standard dose-response curves.

Immunoassays

Radioimmunoassay, fluorescent immunoassay, fluorescent polarization immunoassay, and enzyme immunoassay procedures have all been used to measure antimicrobial agents in serum and other body fluids. The basic principles of these assays are similar in that they all use antibodies directed against the specific antimicrobial agents to be assayed. Because of the nature of these tests, they may be performed in the chemistry or immunology sections of the laboratory. Several commercial manufacturers offer various immunoassay kits for quantifying gentamicin, tobramycin, amikacin, vancomycin, and chloramphenicol assays.

Chromatographic assays

Various chromatographic methods, including gas-liquid, thin-layer, paper chromatography, and high-performance liquid chromatography, have been used on occasion for antimicrobial assays, primarily to measure levels of antimicrobial agents for which commercial immunoassay kits are not available. These tests are performed in research settings only.

POINTS TO REMEMBER

- Antimicrobial susceptibility testing is performed only on bacteria likely causing an infection.
- The CLSI determines standards for antimicrobial susceptibility testing and reporting in clinical laboratories in the United States.
- The FDA has the regulatory authority for setting susceptibility test interpretive criteria and QC parameters.
- Antimicrobial susceptibility testing protocols describing what, when, and how to test and report should be developed with input from clinicians, pharmacists, and others who have

clinical experience with the antimicrobial therapy practices of the institution.

- Selective or cascading reporting refers to reporting broader-spectrum agents only in select situations, such as when the patient's isolate is resistant to narrower-spectrum agents.
- Several variables must be controlled when performing any type of antimicrobial susceptibility test; inoculum standardization is one of the most important of these. Testing too few or too many bacteria can yield erroneous results.
- The disk diffusion, Etest, broth microdilution MIC test, and automated MIC tests are the most common methods currently used for antimicrobial susceptibility testing in clinical laboratories.
- The disk diffusion (or Kirby-Bauer) test is a qualitative method; results are reported as susceptible, intermediate, nonsusceptible, or resistant.
- The MIC test is a semi-quantitative method, and the concentration ($\mu\text{g}/\text{mL}$) of a drug required to inhibit the growth of bacterial isolate is reported together with a *susceptible*, *intermediate*, *nonsusceptible*, or *resistant* interpretation (e.g., ampicillin MIC = $8\mu\text{g}/\text{mL}$; susceptible).
- Routine antimicrobial susceptibility test methods can be modified for testing fastidious bacteria that require supplemental nutrients, modified incubation conditions, or both.
- An oxacillin disk ($1\mu\text{g}$) can be used to screen for penicillin susceptibility in *S. pneumoniae* on isolates not recovered from blood or CSF.
- β -hemolytic streptococci remain universally susceptible to penicillin, the drug of choice for treating infections caused by these organisms, and susceptibility testing is usually not required.
- The CLSI reference method described for testing obligate anaerobic bacteria is agar dilution; however, a broth microdilution method can be used for less fastidious species of anaerobes, such as *Bacteroides* spp.
- The gene most responsible for oxacillin resistance in staphylococci is *mecA*.
- The cefoxitin disk performs better than the oxacillin disk in detecting oxacillin-resistant staphylococci by the disk diffusion method.
- Oxacillin-resistant staphylococci should be considered resistant to all β -lactam agents (including cephalosporins, β -lactam- β -lactamase inhibitor combinations, and carbapenems), despite any susceptible result for these in vitro.
- The D-zone test uses erythromycin to detect inducible clindamycin resistance in staphylococci and β -hemolytic streptococci.
- The vancomycin broth microdilution MIC test or vancomycin agar screen is recommended to detect VISA or VRSA.
- VRE are important healthcare-associated pathogens; however, low-level vancomycin resistance is intrinsic in *E. gallinarum* and *E. casseliflavus*. These differ from true VRE for infection control purposes.
- ESBL-producing bacteria should be considered resistant to all cephalosporins, penicillins, and aztreonam, despite a susceptible result for these in vitro. ESBLs are prevalent in bacteria belonging to the order Enterobacterales.
- Most automated instruments for antimicrobial susceptibility testing are based on turbidimetric detection of bacterial growth or detection of hydrolysis of a fluorogenic growth substrate.
- QC of antimicrobial susceptibility tests is performed with standard reference strains that have defined antimicrobial susceptibility or resistance to the drugs tested.
- Certain species have typical antibiograms that can be used in empiric therapy and to verify the identification and susceptibility results generated on the isolate.
- Cumulative antibiograms represent the percentage of isolates of a given species susceptible to the antimicrobial agents commonly tested against the species.
- Nonsusceptible does not necessarily mean that the organism is resistant to the antimicrobial.
- MBC testing or serum bactericidal testing may be useful in select situations, such as for patients who are immunosuppressed with serious infections or in whom infection is at a site where immune mechanisms are not optimal, e.g., endocarditis.
- MBC testing is performed after completion of a broth dilution MIC test, and the end point is 99.9% killing of the test bacteria.
- Serum bactericidal testing requires use of the patient's serum obtained at appropriate times surrounding dosing of the antimicrobial agent(s), that is, peak and trough, and the bacterium causing the patient's infection.
- Synergism testing of drug combinations can be performed using a time-kill or checkerboard assay described by the CLSI in certain select situations.
- Many clinical laboratories use a *mecA* assay to determine oxacillin susceptibility or resistance in staphylococci and *vanA* and *vanB* assays to detect vancomycin resistance in enterococci.

LEARNING ASSESSMENT QUESTIONS

1. Why would it be inappropriate to perform antimicrobial susceptibility tests on viridans streptococci isolated from a throat culture?
2. The turbidity of a McFarland 0.5 standard corresponds to approximately ____ colony-forming units per milliliter.
3. The ____ disk cannot be used to screen for penicillin susceptibility in *Streptococcus pneumoniae* from sputum.
4. Which method does the Clinical and Laboratory Standards Institute (CLSI) suggest as the most reliable for detecting oxacillin resistance in staphylococci?
 - a. Oxacillin disk diffusion test
 - b. Cefoxitin disk diffusion test
 - c. Penicillin minimal inhibitory concentration (MIC) test
 - d. Oxacillin agar screen
5. What does the *mecA* gene code for in staphylococci?
 - a. β -Lactamase and penicillin resistance
 - b. Altered penicillin-binding protein and vancomycin resistance
 - c. Penicillin-binding protein 2a and oxacillin resistance
 - d. β -Lactamase and oxacillin resistance
6. With which of the following profiles should *S. aureus* or β -hemolytic streptococci be subjected to in D-zone testing?
 - a. Oxacillin-resistant
 - b. Penicillin-resistant
 - c. Erythromycin-resistant and clindamycin-resistant
 - d. Erythromycin-resistant and clindamycin-susceptible
7. An ampicillin-susceptible *Enterococcus faecalis* from a blood culture has high-level resistance to the aminoglycoside gentamicin. Which of the following statements is true?
 - a. Ampicillin and gentamicin will be synergistic.
 - b. Ampicillin and gentamicin will not be synergistic.

- c. Penicillin and gentamicin will be synergistic.
- d. Cefazolin and gentamicin will be synergistic.
8. Extended-spectrum β -lactamase-producing isolates should be considered resistant to which of the following agents?
 - a. Cephalosporins, penicillins, and aztreonam
 - b. Cephalosporins, penicillins, and aminoglycosides
 - c. Cephalosporins, penicillins, and β -lactamase inhibitors
 - d. Cefotaxime-clavulanic acid and ceftazidime-clavulanic acid
9. Which of the following organisms is commonly tested for β -lactamase production?
 - a. *Neisseria meningitidis*
 - b. *Klebsiella pneumoniae*
 - c. *Streptococcus pneumoniae*
 - d. *Haemophilus influenzae*
10. Which of the following statements is true about quality control (QC) testing of antimicrobial susceptibility tests?
 - a. A laboratory must perform QC every day that patient isolates are tested.
 - b. A laboratory can perform QC weekly if it performs fewer than 10 tests daily.
 - c. A laboratory can perform QC weekly once accurate performance of 20 to 30 days of daily QC has been documented.
 - d. A laboratory does not have to perform QC if an automated test antimicrobial susceptibility test system is used.
11. Identify each of the following statements as true or false.
 - a. Ampicillin susceptibility in *Pseudomonas aeruginosa* is unusual. ____
 - b. Many *Staphylococcus aureus* strains are vancomycin resistant. ____
 - c. Penicillin is the drug of choice for treating gonorrhea. ____
 - d. *Stenotrophomonas maltophilia* strains are usually susceptible to trimethoprim-sulfamethoxazole. ____
 - e. *Escherichia coli* strains are expected to be resistant to all aminoglycosides. ____
12. Which of the following statements is true?
 - a. An organism that is reported as nonsusceptible to an antimicrobial is definitely resistant to the antimicrobial.
 - b. The term nonsusceptible is used when the interpretive categories of intermediate and resistant do not apply.
 - c. The organization that oversees preparing technical documents for international use is the European Medicines Agency.
 - d. The *vanA* gene codes for chloramphenicol resistance.
13. Why is a bactericidal drug regimen necessary for treating patients with bacterial endocarditis?
14. The MBC end point is the lowest concentration of antimicrobial agent that kills --- of the test bacteria.
15. It is important to test bacteria in the --- phase of growth when performing tests to assess bactericidal activity.
16. Which of the following definitions best describes synergism?
 - a. The activity of the drug combination is greater than that of the individual agents.
 - b. The activity of the drug combination is less than that of the individual agents.
 - c. The activity of the drug combination is equal to that of the individual agents.
 - d. The test organism is resistant to both drugs in the combination.
17. Why is the use of molecular assays to detect antimicrobial resistance genes limited?
 - a. Genes are not responsible for most types of antimicrobial resistance.
 - b. Genes may be present but may not be expressed; therefore the presence of the gene does not always correlate with resistance.
 - c. Researchers have been unable to identify genes for antimicrobial resistance.
 - d. Testing is too expensive.
18. Which of the following classes of antimicrobial agents poses the greatest toxicity risks and therefore is frequently monitored using antimicrobial assays?
 - a. Cephalosporins
 - b. Sulfonamides
 - c. Aminoglycosides
 - d. Tetracyclines
19. The presence of the *blaZ* gene is expected to confer resistance to which of the following?
 - a. Cephalosporins only
 - b. Erythromycin
 - c. Oxacillin
 - d. Several β -lactam antibiotics

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PART 2

Laboratory identification of significant isolates

Staphylococcus and similar organisms

Lindsey E. Nielsen and Jed M. Doxtater

CHAPTER OUTLINE

General characteristics, 309

Clinically significant species, 310

Staphylococcus aureus, 310

Staphylococcus epidermidis, 314

Staphylococcus saprophyticus, 314

Staphylococcus lugdunensis, 314

Other coagulase-negative staphylococci, 314

Laboratory diagnosis, 315

Specimen collection and handling, 315

Microscopic examination, 315

Isolation and identification, 315

Antimicrobial susceptibility, 319

Methicillin-resistant staphylococci, 320

Vancomycin-resistant staphylococci, 321

Macrolide resistance, 322

Bibliography, 323

OBJECTIVES

After reading and studying this chapter, you should be able to:

1. Describe the general characteristics of the genus *Staphylococcus*.
2. Differentiate the characteristics of staphylococci with those of other gram-positive cocci.
3. Describe the virulence factors associated with *Staphylococcus* spp. and their role in pathogenesis.
4. Compare the clinical infections associated with various staphylococcal species and the populations at greatest risk.
5. Evaluate the slide coagulase, tube coagulase, and agglutination tests for the identification of *Staphylococcus aureus*.
6. Develop a diagnostic testing algorithm to differentiate among clinically relevant *Staphylococcus* species.
7. Discuss the characteristics that should be used to identify a *Staphylococcus*-like organism isolated from a clinical sample.
8. Explain why oxacillin resistance among *S. aureus* isolates is a serious clinical problem.

9. Critique the current practices in detecting *S. aureus* resistance to oxacillin, clindamycin, and vancomycin.
10. Describe when testing with ceftiofur or testing for *mecA* or penicillin-binding protein 2a would be used.
11. Evaluate the role of present and possible future vancomycin resistance in *S. aureus* in infection control compared with that in methicillin-resistant *S. aureus* (MRSA).
12. Evaluate the role for molecular testing of MRSA among different specimen types and patient populations.
13. Explain how matrix-assisted laser desorption/ionization–time-of-flight (MALDI-TOF) mass spectrometry might be used in clinical microbiology laboratories to identify *Staphylococcus* spp. and similar organisms.

KEY TERMS

α -Hemolysin

β -Hemolysin

β -Lactamases

Borderline oxacillin-resistant

Staphylococcus aureus
(BORSA)

Bullous impetigo

Carbuncles

Catalase

Clumping factor

Coagulase

Coagulase-negative staphylococci
(CoNS)

Community-associated
methicillin-resistant
Staphylococcus aureus
(CA-MRSA)

Cytolytic toxins

Decolonization

D-zone test

Enterotoxins

Exfoliative toxins

Folliculitis

Furuncles

Hospital-associated methicillin-
resistant *Staphylococcus*
aureus (HA-MRSA)

Impetigo

Matrix-assisted laser desorp-
tion/ionization–time-of-flight
(MALDI-TOF)

Methicillin-resistant
Staphylococcus aureus
(MRSA)

Methicillin-resistant
Staphylococcus epidermidis
(MRSE)

Methicillin-susceptible
Staphylococcus aureus
(MSSA)

Osteomyelitis

Panton-Valentine leukocidin (PVL)

Penicillin-binding protein (PBP)

Protein A

Pyroglutonyl arylamidase (PYR)

Ritter disease

Scalded skin syndrome (SSS)
 Small colony variants (SCVs)
 Staphylocoagulase
 Toxic shock syndrome (TSS)
 Toxic shock syndrome toxin-1 (TSST-1)

Vancomycin-intermediate
Staphylococcus aureus (VISA)
 Vancomycin-resistant
Staphylococcus aureus (VRSA)

Case in point

During track practice, a 15-year-old female high school student tripped over a barrier and heard a “pop” in her right knee cap. She experienced pain in her right knee and shin and was not able to walk on that leg. The patient was seen at an emergency clinic, where it was determined that she may have torn her anterior cruciate ligament (ACL), and she was referred to an orthopedic surgeon. The damaged ACL was removed and replaced with an ACL graft, which was secured in place with screws. She was given a brace to wear for several weeks, given broad-spectrum therapy, and was to be followed up by her primary care physician within a few weeks.

During the second week of recovery, increased pain was noted in the injured knee, and a clear exudate was noted at the incision site. The site was drained, and primary cultures and antimicrobial susceptibility tests were performed at a large metropolitan laboratory. Gram-stain results showed many gram-positive cocci in pairs and clusters with few polymorphonuclear neutrophils (PMNs). Heavy growth of coagulase-positive *Staphylococcus* species grew at 18 hours. While validating a **matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF)** mass spectrometry procedure, an analysis was performed on the isolate. The MALDI-TOF report did not identify the organism as *Staphylococcus aureus*. The preliminary report was released as staphylococci species based on the MALDI-TOF identification with susceptibility results using the latest reference ranges for minimum inhibitory concentration (MIC) interpretation for this species of staphylococci. A review of the patient’s history showed that the student and her sister were frequent volunteers at a local animal shelter. The isolate, which was referred to the state health department, was identified as *Staphylococcus pseudintermedius*.

Issues to consider

After reading the patient’s case history, consider:

- Risk factors associated with acquiring this organism
- The methods that were used to identify this isolate
- Coagulase methods for staphylococci (slide, tube, and rapid) and their limitations
- The role that MALDI-TOF mass spectrometry can have in identifying common and uncommon species
- The importance of correct identification and using the latest susceptibility guidelines

Gram-positive cocci are common isolates in the clinical microbiology laboratory. Although most gram-positive cocci are members of the indigenous microbiota, some species are causative agents of serious infectious disease. This chapter discusses commonly encountered staphylococci, their characteristics, the infections they produce, and their laboratory identification. Infections caused by *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*,

Staphylococcus lugdunensis, and *Staphylococcus haemolyticus* are emphasized.

General characteristics

The staphylococci are **catalase-positive**, gram-positive cocci. On stained smears, they exhibit spherical cells (0.5 to 1.5 µm) that appear singly, in pairs, and in clusters. The genus name, *Staphylococcus*, is derived from the Greek term *staphle*, meaning “bunches of grapes.” Although the Gram stain can be characteristic of staphylococci, microscopy alone cannot differentiate staphylococci from other gram-positive cocci. Staphylococci are members of the family Staphylococcaceae. The staphylococci are nonmotile, non-spore-forming, and aerobic or facultatively anaerobic, although a few strains are obligate anaerobes. Colonies produced after 18 to 24 hours of incubation are medium size (4 to 8 mm) and appear cream-colored, white, or rarely light gold, and “buttery-looking.”

Rare strains of staphylococci are fastidious, requiring carbon dioxide, hemin, or menadione for growth. These so-called **small colony variants (SCVs)** grow on media containing blood, forming colonies about one tenth the size of wild-type strains, even after 48 hours or more of incubation. Some species are β-hemolytic. Staphylococci are common isolates in the clinical laboratory and are responsible for numerous suppurative infections. These organisms are normal inhabitants of the skin and mucous membranes of humans and other animals.

The staphylococci resemble some members of the family Micrococcaceae, such as the genus *Micrococcus*. Micrococci are catalase-producing, coagulase-negative, gram-positive cocci found in the environment and as members of the indigenous skin microbiota. They are often recovered with staphylococci and can be differentiated easily from **coagulase-negative staphylococci (CoNS)** with the characteristics listed in [Table 14.1](#). Some micrococci have a tendency to produce a yellow pigment ([Fig. 14.1](#)). Other catalase-positive, gram-positive cocci that are occasionally recovered from human clinical specimens include *Rothia mucilaginosa* and *Alloicoccus otitis* (recovered from human middle ear fluid).

Staphylococcal species can be initially differentiated by the **coagulase** test (see procedure in [Appendix D](#) on Evolve); a positive test result is a clot formed in a tube containing plasma that is caused by staphylocoagulase. The staphylocoagulase-producing (coagulase-positive) staphylococci are *S. aureus*, *S. intermedius*, *S. pseudintermedius*, *S. hyicus*, *S. delphini*, *S. lutrae*, *S. agnetis*, and some strains of *S. schleiferi*. Isolates such as *S. lugdunensis* and *S. schleiferi* also can be occasionally mistaken for coagulase-positive staphylococci because of the presence of **clumping factor**. Clumping factor causes bacterial cells to agglutinate in plasma and was the basis of a test known as the *slide coagulase test*. This is considered an obsolete test, and only the tube coagulase test should be used for definitive testing. Rapid latex and hemagglutination assays provide presumptive identification of *S. aureus*. The principles of these tests are discussed later. The majority of clinical staphylococcal isolates that are tube coagulase positive are *S. aureus*.

The other staphylococcal species that can be positive with the traditional coagulase test are often animal-associated species and are less frequently isolated. A review of the patient

Table 14.1 Differentiation between staphylococci and micrococci in the routine laboratory

Test	Staphylococci	Micrococci
Modified oxidase ^a	–	+
Anaerobic acid production from glucose	+	–
Growth on Furoxone–Tween 80–oil red O agar	–	+
Anaerobic acid production from glycerol in the presence of erythromycin	+	–
Resistance to bacitracin (0.04 units)	R ^b	S
Lysosome (50-mg disk)	R	S
Lysostaphin test	S ^b	R

^aCommercially available, useful for presumptive identification.^bSome stains show opposite reaction.

R, Resistant; S, sensitive.

Modified from Schumacher-Perdreau, F. (1991). Clinical significance and laboratory diagnosis of coagulase-negative staphylococci. *Clin Microbiol News*, 13, 97.

Fig. 14.1 Micrococci growing on sheep blood agar showing yellow pigment.

history and antimicrobial susceptibility pattern can be helpful in differentiating these less frequently isolated staphylococci species from *S. aureus*. The use of manual and automated commercial systems for identification has become routine in many laboratories. Referral to reference laboratories might be required for unusual isolates on consultation with the attending physician.

S. aureus is the most clinically significant species. It causes various cutaneous infections and purulent abscesses. These skin and soft tissue infections can be superficial, such as impetigo or cellulitis. Cutaneous infections can progress to deeper abscesses, such as **carbuncles**; involve other organ systems; and produce bacteremia and septicemia. *S. aureus* is a common cause of infective endocarditis and toxin-induced

diseases, such as food poisoning, and is associated with **scalded skin syndrome (SSS)** and **toxic shock syndrome (TSS)**.

Staphylococci that do not produce coagulase are referred to as *coagulase-negative staphylococci* (CoNS). The most clinically significant species in this group are *S. epidermidis*, *S. saprophyticus*, *S. lugdunensis*, and *S. haemolyticus*. *S. epidermidis* has been known to cause various healthcare-acquired or nosocomial infections, whereas *S. saprophyticus* is associated mainly with urinary tract infections (UTIs), predominately in adolescent girls and young women. *S. haemolyticus* is occasionally recovered from wounds, septicemia, UTIs, and native valve infections. *S. lugdunensis*, like *S. aureus*, is slide coagulase positive, so further testing, including the tube coagulase test, becomes crucial in differentiation. *S. lugdunensis* can be an aggressive pathogen and has been associated with catheter-related bacteremia and endocarditis. Because of reporting criteria, *S. lugdunensis* should be identified to the species level to provide the correct treatment options when reporting antimicrobial susceptibilities.

Currently more than 40 recognized species and subspecies of CoNS exist. Those species that have been isolated from humans are usually associated with the skin and mucous membranes; however, some are found in very specific sites, such as the head (*S. capitis*) or ear (*S. auricularis*). Some are found only in animals, but others have been associated with human disease (Table 14.2).

Clinically significant species

Staphylococcus aureus

S. aureus is responsible for numerous infections, ranging from relatively mild to life-threatening. Infections can be categorized as suppurative or toxin-mediated disease. *S. aureus* can be recovered from almost any clinical specimen and is an important cause of healthcare-associated infections. *S. aureus* is an important community-acquired pathogen, and the increasing drug resistance is a concern with this common isolate.

Virulence factors

The pathogenicity associated with *S. aureus* can be attributed to numerous virulence factors (Table 14.3), including **enterotoxins**, **cytolytic toxins**, and cellular components such as **protein A**. Several cytolytic toxins and **exfoliative toxins** have been identified. Despite these virulence factors, innate resistance to *S. aureus* is fairly high, and the organism is regarded as an opportunistic pathogen.

Enterotoxins

Staphylococcal enterotoxins are heat-stable exotoxins that cause symptoms, including diarrhea and vomiting. Numerous serologically distinct enterotoxins have been identified with the majority falling into groups A to E and G to J. These toxins are produced by 30% to 50% of *S. aureus* isolates. Because the enterotoxins are stable at 100° C for 30 minutes, reheating contaminated food does not prevent disease. Staphylococcal food poisoning is most commonly

Table 14.2 Coagulase-negative staphylococci and their clinical source and significance

Staphylococcus species	Source
S. epidermidis group	
<i>S. epidermidis</i>	Human ^a , animal
<i>S. haemolyticus</i>	Human ^a
<i>S. hominis</i>	Human
<i>S. capitis</i> subsp. <i>capitis</i>	Human
<i>S. capitis</i> subsp. <i>ureolyticus</i>	Human
<i>S. caprae</i>	Human, animal
<i>S. auricularis</i>	Human
<i>S. saccharolyticus</i>	Human
<i>S. warneri</i>	Human, animal
<i>S. pasteurii</i>	Animal, human
S. saprophyticus group	
<i>S. saprophyticus</i>	Human ^a
<i>S. cohnii</i> subsp. <i>cohnii</i>	Animal, human
<i>S. cohnii</i> subsp. <i>urealyticum</i>	Animal, human
<i>S. nepalensis</i>	Animal, human
<i>S. xylosus</i>	Animal
<i>S. arlettae</i>	Animal
<i>S. equorum</i>	Animal, human
<i>S. gallinarum</i>	Animal
<i>S. kloosii</i>	Animal
S. simulans group	
<i>S. simulans</i>	Animal, human
<i>S. carnosus</i>	Animal
S. intermedius group	
<i>S. schleiferi</i> subsp. <i>schleiferi</i>	Animal ^a , human
S. sciuri group	
<i>S. sciuri</i>	Animal, human
<i>S. lentus</i>	Animal, human
<i>S. fleuretti</i>	Animal, human
<i>S. stepanovicii</i>	Animal, human
<i>S. vitulinus</i>	Animal, human
S. hyicus group	
<i>S. chromogenes</i>	Animal
Unspecified group	
<i>S. condimenti</i>	Animal
<i>S. felis</i>	Animal
<i>S. hyicus</i>	Animal
<i>S. lugdunensis</i>	Human ^a , animal
<i>S. muscae</i>	Animal
<i>S. piscifermentans</i>	Animal
<i>S. pseudintermedius</i>	Animal

^aCommon human or veterinary disease.

caused by enterotoxins A, B, and D. Enterotoxins B and C and sometimes G and I are associated with TSS. Enterotoxin B has been linked to staphylococcal pseudomembranous enterocolitis. These toxins, along with **toxic shock syndrome toxin-1** (TSST-1), are superantigens and have the ability to interact with many T cells, activating an aggressive, overre-active immune response.

Toxic shock syndrome toxin-1

Previously referred to as *enterotoxin F*, TSST-1 is a chromosomal-mediated toxin that causes the majority of cases of menstruating-associated TSS and approximately 50% of non-menstruating cases. TSST-1 is a superantigen, stimulating T-cell proliferation and the subsequent production of a large number of cytokines that are responsible for the symptoms. At low concentrations, TSST-1 causes leakage by endothelial cells, and it is cytotoxic to these cells at higher concentrations. TSST-1 is absorbed through the vaginal mucosa, leading to the systemic effects seen in TSS associated with tampon use.

Exfoliative toxin

Exfoliative toxin is also known as *epidermolytic toxin*. There are two types of toxins denoted simply as *exfoliative toxin A* and *exfoliative toxin B*. They cause the epidermal layer of the skin to slough off and are known to cause staphylococcal SSS, sometimes referred to as **Ritter disease**. Ritter disease is most common in newborns and infants, with most cases reported in children younger than 5 years of age. This toxin has also been implicated in **bullous impetigo**.

Cytolytic toxins

S. aureus produces other extracellular proteins that affect red blood cells and leukocytes. These hemolysins and leukocidins are cytolytic toxins with properties different from the properties of previously described toxins. *S. aureus* produces four hemolysins: alpha, beta, gamma, and delta. **α-Hemolysin**, in addition to lysing erythrocytes, can damage platelets and macrophages and cause severe tissue damage. **β-Hemolysin** (sphingomyelinase C) acts on sphingomyelin in the plasma membrane of erythrocytes and is also called the “hot-cold” *lysin*. The “hot-cold” feature associated with this toxin is seen as enhanced hemolytic activity on incubation at 37° C and subsequent exposure to cold (4° C). This hemolysin is exhibited in the Christie, Atkins, Munch-Petersen (CAMP) laboratory test to identify group B streptococci. **δ-Hemolysin**, although found in a higher percentage of *S. aureus* strains and some CoNS, is considered less toxic to cells than either α-hemolysin or β-hemolysin. **γ-Hemolysin** is found only in association with **Panton-Valentine leukocidin** (PVL).

PVL is an exotoxin lethal to polymorphonuclear leukocytes. It has been implicated as contributing to the invasiveness of the organism by suppressing phagocytosis and has been associated with severe cutaneous infections and necrotizing pneumonia. Although produced by relatively few strains of *S. aureus*, it is often associated with community-acquired staphylococcal infections and might be a marker for such infections.

Enzymes

Several enzymes are produced by staphylococci. Examples include coagulase, protease, hyaluronidase, and lipase.

Table 14.3 Virulence factors of *Staphylococcus aureus*

Hemolysins	
α	Damages erythrocytes, platelets, and macrophages
β	Also known as <i>sphingomyelinase C</i> ; disrupts the erythrocyte plasma membranes. Responsible for CAMP assay effectiveness.
δ	Found in some CoNS strains as well as in <i>S. aureus</i> . Less toxic than other hemolysins.
γ	Associated with Pantone-Valentine leukocidin.
Pantone-Valentine leukocidin	Polymorphonuclear leukocyte toxicity
β-Lactamase	Enzyme that cleaves the ring structure of penicillins and derivative antibiotics, making them ineffective.
Penicillin-binding protein 2	Altered membrane binding protein resulting in resistance to some β-lactam antibiotics.
Hyaluronidase	Permits bacteria to spread through connective tissues.
Lipases	Common to <i>S. aureus</i> and CoNS. Degrades lipids on skin surface, making it more susceptible to bacterial entry into epidermal layers.
Staphylocoagulase	Responsible for a positive tube coagulase test result. Also present in <i>S. intermedius</i> , <i>S. pseudintermedius</i> , <i>S. hyicus</i> , <i>S. delphini</i> , <i>S. lutrae</i> , <i>S. agnetis</i> , and some <i>S. schleiferi</i> .
Toxic shock syndrome toxin-1	A superantigen causing an overreactive immune response. Formerly known as <i>enterotoxin F</i> .
Protein A	Binds IgG and prevents phagocytosis.
Enterotoxins A–E, G, J	Enterotoxins A, B, and D are the cause of the majority of staphylococcal food poisoning cases; heat stable. Enterotoxins B and C, and rarely G and I, can cause enterocolitis.
Exfoliative toxins	Also known as <i>epidermolytic toxin</i> . Types A and B. Solely responsible for SSS and present in a minority of <i>S. aureus</i> species. May also cause bullous impetigo.

CAMP, Christie, Atkins, Munch-Petersen; CoNS, coagulase-negative staphylococcus; IgG, immunoglobulin G; SSS, scalded skin syndrome.

Staphylocoagulase is produced mainly by *S. aureus*. Although the exact role of coagulase in pathogenicity is uncertain, it is considered a virulence marker. Many strains of *S. aureus* produce hyaluronidase. This enzyme hydrolyzes hyaluronic acid present in the intracellular ground substance that makes up connective tissues, permitting the spread of bacteria during infection. Lipases are produced by both coagulase-positive staphylococci and CoNS. Lipases act on lipids present on the surface of the skin, particularly fats and oil secreted by the sebaceous glands. Protease, lipase, and hyaluronidase are capable of destroying tissue and may facilitate the spread of infection to adjoining tissues.

Protein A

Protein A is one of several cellular components that have been identified in the cell wall of *S. aureus*. Probably the most significant role of protein A in infections caused by *S. aureus* is its ability to bind the Fc portion of immunoglobulin G (IgG). Binding IgG in this manner can block phagocytosis and negate the protective effects of IgG.

Epidemiology

The primary reservoir for staphylococci is the human nares, with colonization also occurring in the vagina, pharynx, axillae, and other skin surfaces. Nasal carriage in patients admitted to the hospital is common. Because contact among patients and hospital personnel is not unusual, transfer of organisms often occurs. Consequently, increased colonization in patients and hospital workers occurs frequently. Hospital outbreaks can develop in patients in nurseries and burn units and among patients who have undergone surgery or other invasive procedures. Acute care facilities are encouraged to implement strategies to prevent hospital-onset *S. aureus* bloodstream

infections. Infection control practices including hand hygiene, environmental cleaning and disinfection, and use of personal protective equipment should be routinely enforced.

In the community, intravenous drug users were estimated to be 16.3 times more likely to acquire invasive **methicillin-resistant *Staphylococcus aureus* (MRSA)** infections than others. Between 2011 and 2016, cases of invasive MRSA from injecting drugs increased from 4.1% to 9.2%. Increases in non-sterile drug injection, which can lead to increases in MRSA infections, emphasize the significance of public health interventions to prevent such infections.

Transmission of *S. aureus* may occur by direct contact with unwashed, contaminated hands and by contact with inanimate objects (fomites). Both healthcare- and community-acquired infections caused by MRSA are a major health care concern. **Decolonization** protocols have been introduced to reduce colonization for specific populations, such as patients in the intensive care unit.

Infections caused by *Staphylococcus aureus*

As with most infections, the development of staphylococcal infection is determined by the virulence of the strain, size of the infectious dose, and status of the host's immune system. Infections are initiated when a breach of the skin or mucosal barrier allows staphylococci access to adjoining tissues or the bloodstream. Any event that compromises the host's ability to resist infection encourages colonization and infection. Individuals with normal defense mechanisms are able to combat the infection more easily than individuals with impaired immune systems. Once the organism has crossed the initial barriers, it activates the host's acute inflammatory response, which leads to the proliferation and activation of polymorphonuclear cells. However, the organisms are able to

resist the action of inflammatory cells by the production of toxins and enzymes, establishing a focal lesion.

Skin and wound infections

Infections caused by *S. aureus* are suppurative. Typically, the abscess is filled with pus and surrounded by necrotic tissues and damaged leukocytes. Some common, more benign skin infections caused by *S. aureus* include **folliculitis**, **furuncles**, and **bullous impetigo**. These opportunistic infections usually occur as a result of previous skin injuries, such as cuts, burns, and surgical incisions. Folliculitis is a relatively mild inflammation of a hair follicle or oil gland; the infected area is raised and red. Furuncles (boils), which can be an extension of folliculitis, are large, raised, superficial abscesses.

Carbuncles occur when larger, more invasive lesions develop from multiple furuncles, which can progress into deeper tissues. In contrast with patients with furuncles, patients with carbuncles often present with fever and chills, indicating systemic spread of the bacteria. Bullous impetigo caused by *S. aureus* differs from streptococcal nonbullous **impetigo**, also known as *impetigo contagiosa*, in that staphylococcal pustules are larger and surrounded by a small zone of erythema. Impetigo is a highly contagious infection that is easily spread by direct contact, fomites, or autoinoculation.

Staphylococcal infections also can be secondary to skin diseases of different causes. Dry, irritated skin combined with poor personal hygiene encourages the development of infection. Some of these infections are manifested because of increased colonization of the organisms in blocked hair follicles, sebaceous glands, and sweat glands. Occasionally, these superficial infections can be misidentified as insect or spider bites. Immunocompromised individuals—particularly patients who are receiving chemotherapy, are debilitated by chronic illnesses, or have invasive devices implanted—are predisposed to developing staphylococcal infections. Infections of the skin and soft tissues can progress in seriousness and become life-threatening.

Scalded skin syndrome

SSS is a bullous exfoliative dermatitis that occurs primarily in newborns and previously healthy young children. This syndrome is caused by staphylococcal exfoliative or epidermolysin toxin produced by *S. aureus*, which is probably present at a lesion distant from the site of exfoliation. The disease has also been recognized in adults. Cases of SSS in adults occur most commonly in patients with chronic renal failure and in patients with compromised immune systems. Although the mortality rate is low (0% to 7%) in cases seen among children, the rate in adults can be 50%.

The severity of the disease ranges from a localized skin lesion in the form of a few blisters, pemphigus neonatorum, to a more extensive generalized condition affecting 90% of the body, known as *Ritter disease*. Localized lesions contain purulent material. This lesion can progress to the generalized form, which is characterized by cutaneous erythema followed by profuse peeling of the epidermal layer of the skin. The typical pattern in which the erythema occurs is origination from the face, neck, axillae, and groin and then extension to the trunk and extremities. The duration of the disease is brief, about 2 to 4 days, with complete healing occurring after around 10 days. The incidence of spontaneous recovery among children

is high, and there is scarring. The toxin is metabolized and excreted by the kidneys. It is believed that an immature or compromised renal or immune system contributes to the higher incidence of SSS among children younger than 5 years of age and among adults.

Staphylococcal SSS must be differentiated from the clinically similar toxic epidermal necrolysis (TEN), a serious, potentially fatal disease. TEN has multiple causes but is most commonly associated with drug reactions, and it has been linked to antimicrobials and anticonvulsives. The cause is unknown, but symptoms appear to be caused by a hypersensitivity reaction. Although it has a very similar initial presentation to that of SSS, treatments differ. TEN can be resolved by administration of steroids early in the initial stages of presentation, whereas steroids aggravate SSS. The mortality rate associated with TEN is high, and administration of suspected offending drugs should be stopped as soon as possible.

Toxic shock syndrome

TSS is a rare but potentially fatal, multisystem disease characterized by a sudden onset of fever, chills, vomiting, diarrhea, muscle aches, and rash, which can quickly progress to hypotension and shock. It was first described in 1978 and was associated with women using highly absorbent tampons, although some cases appeared in men, children, and nonmenstruating women. The two categories of TSS are menstruating-associated and nonmenstruating-associated. Although nonmenstruating TSS has been associated with nearly any staphylococcal infection, many cases have been seen with postsurgical infections.

Staphylococcal TSS generally results from a localized infection by *S. aureus*; only the toxin TSST-1 is systemic. The initial clinical presentation of TSS consists of high temperature, rash, and signs of dehydration, particularly if the patient has had watery diarrhea and vomiting for several days. In extreme cases, patients may be severely hypotensive and in shock. The rash is found predominantly on the trunk but can spread over the entire body. Cultures of focal lesions may yield *S. aureus*, but blood cultures are often negative. *S. aureus* does not need to be isolated to confirm the diagnosis of TSS. Supportive therapy to replace vascular volume loss is given, along with appropriate antimicrobial therapy. Most patients with TSS recover, although 2% to 5% of the cases may be fatal. The use of minimum absorbency tampons and warning label requirements from the U.S. Food and Drug Administration (FDA) for tampon products have greatly decreased the risk of TSS.

Food poisoning

S. aureus enterotoxins, most commonly A (78%), D (38%), and B (10%), have been associated with gastrointestinal disturbances. The source of contamination is usually an infected food handler. Staphylococcal food poisoning is a type of intoxication resulting from ingestion of a toxin formed outside the body. Disease occurs when food becomes contaminated with enterotoxin-producing strains of *S. aureus* by improper handling and storage, which allows growth of the bacteria and resulting toxin production. An individual ingests the food contaminated with enterotoxin and becomes ill.

Foods that are often incriminated in staphylococcal food poisoning include salads, especially salads containing

mayonnaise and eggs; meat or meat products; poultry; egg products; bakery products with cream fillings; sandwich fillings; and dairy products. Foods kept at room temperature are especially susceptible to higher levels of toxin production when contaminated with toxin-producing staphylococci. The enterotoxins do not cause any detectable odor or change in the appearance or taste of the food. Symptoms appear rapidly (approximately 2 to 8 hours after ingestion of the food) and resolve within 24 to 48 hours. Although no fever is associated with this condition, nausea, vomiting, abdominal pain, and severe cramping are common. Diarrhea and headache can also occur. Death from staphylococcal food poisoning is rare, although such cases have occurred among older adult patients, infants, and severely debilitated individuals.

Other infections

Staphylococcal bacteremia has been observed among intravenous drug users. The organisms gain entrance to the bloodstream via contaminated needles or from a focal lesion present on the skin or in the respiratory or genitourinary tract. Any local *S. aureus* infection can progress to a bacteremia leading to a secondary pneumonia, endocarditis, or bone infection. Besides producing acute, rapidly progressive disease, *S. aureus* can cause chronic infections. Often, these chronic infections are associated with SCVs. These variants are adapted for intracellular growth and are more difficult to detect on laboratory media.

Staphylococcal pneumonia has been known to occur secondary to influenza virus infection. Although relatively rare, staphylococcal pneumonia has a high mortality rate. Pneumonia, which develops as a contiguous, lower respiratory tract infection or a complication of bacteremia, is characterized by multiple abscesses and focal lesions in the pulmonary parenchyma. Infants and immunocompromised individuals, such as older adults and patients receiving chemotherapy or immunosuppressants, are most affected.

S. aureus is also associated with a number of bone infections including osteomyelitis, septic arthritis, and prosthetic joint infections. Staphylococcal osteomyelitis occurs as a manifestation secondary to bacteremia. Bacteria may lodge in the diaphysis of the long bones and establish an infection. Symptoms include fever, chills, swelling, and pain around the affected area. Septic arthritis is frequently caused by *S. aureus* in children, especially with trauma to the extremities. Septic arthritis can also occur in patients with a history of rheumatoid arthritis, diabetes mellitus, recent joint surgery, skin infections, or intravenous drug abuse. The organisms may or may not be recovered from aspirated joint fluid.

Staphylococcus epidermidis

The role of *S. epidermidis* as an etiologic agent of disease is evident. *S. epidermidis* is considered normal skin biota but is a common source of hospital-acquired infections and often a contaminant in improperly collected blood culture specimens. Some predisposing factors for hospital-acquired infection include instrumentation procedures such as catheterization, medical implantation, and immunosuppressive therapy. *S. epidermidis* infections have been associated with intravascular catheters, cerebrospinal fluid shunts, and other prosthetic devices. *S. epidermidis* is also a common cause of healthcare-acquired UTIs. Prosthetic valve endocarditis is most commonly

caused by *S. epidermidis*, although other CoNS, such as *S. lugdunensis*, have also been recovered in these cases. Septicemia has been reported in immunocompromised patients.

Infections associated with the use of implants, such as indwelling catheters and prosthetic devices, are often caused by isolates shown to produce a biofilm. Biofilm production is a key component in bacterial pathogenesis and is a complex interaction among the host, the indwelling device, and bacteria (see Chapter 31). One bacterial factor involved in adherence of *S. epidermidis* may be poly(γ -DL-glutamic acid), which provides a protective advantage against host defenses.

Staphylococcus saprophyticus

S. saprophyticus is associated with UTIs in young women. It is the second most common cause, after *E. coli*, of uncomplicated cystitis in this population. This species adheres more effectively to the epithelial cells lining the urogenital tract than other CoNS. It is rarely found on other mucous membranes or skin surfaces. When present in urine cultures, *S. saprophyticus* may be found in low numbers (<10,000 colony-forming units per milliliter) and still be considered significant.

Staphylococcus lugdunensis

S. lugdunensis can cause both community-associated and hospital-acquired infections. This organism is more virulent than others known to contain the *mecA* gene, which encodes oxacillin resistance. It is an important pathogen in infective endocarditis, septicemia, meningitis, skin and soft tissue infections, UTIs, and septic shock. Endocarditis caused by *S. lugdunensis* is particularly aggressive, frequently requiring valve replacement, and infections have a high mortality rate. The pathogenicity of this organism more closely resembles *S. aureus* than the CoNS.

Other coagulase-negative staphylococci

Species less commonly seen but established as opportunistic pathogens include *S. haemolyticus*, *S. warneri*, *S. capitis*, *S. simulans*, *S. hominis*, and *S. schleiferi*. A wide range of infections have been associated with these organisms, including endocarditis, septicemia, and wound infections. Other species of CoNS are found as normal biota in humans and animals. Although they are not commonly seen as pathogens, their role in some infections is well established, and they cannot be automatically discarded as contaminants.

S. haemolyticus is a commonly isolated CoNS. It has been reported in wounds, bacteremia, endocarditis, and UTIs. Vancomycin resistance exists in some *S. haemolyticus* isolates. Recently, *S. pseudintermedius*, a common cause of pyoderma in dogs and skin, ear, and postoperative infections in dogs and cats, has been linked to human infections. The first human case was endocarditis in a patient after implantation of a cardioverter-defibrillator device. Subsequently, other human infections reported sporadically have included surgical site infections, rhinosinusitis, and catheter-associated bacteremia. Veterinary staff and pet owners are at risk of zoonotic transmission of *S. pseudintermedius*. Many isolates contain two SCC*mec* elements (II and III) resulting in oxacillin resistance (see later). In addition, resistance to several other classes of antibiotics has been noted.

Case check 14.1

S. pseudintermedius is an opportunistic pathogen of dogs and cats commonly associated with skin, ear, and postoperative infections. This bacterium has recently been associated with a variety of infections in humans, including surgical wounds. In the Case in Point, the patient had a history of volunteering at a local animal shelter.

Laboratory diagnosis

Specimen collection and handling

Proper specimen collection, transport, and processing are essential elements in the correct diagnosis and interpretation of any bacterial culture result. Clinical materials collected from infected sites should be transported to the laboratory without delay to prevent drying, maintain the proper environment, and minimize the growth of contaminating organisms. Although the recovery of staphylococci requires no special procedures, specimens should be taken from the site of infection after appropriate cleansing of the surrounding area to avoid contamination by the skin microbiota. Normal skin biota contamination can be further reduced by the physician submitting secretion aspirates, tissue samples, or blood culture specimens instead of swabs.

Microscopic examination

Microscopic examination of stained smears prepared directly from clinical samples (Fig. 14.2) provides information that is helpful in the early diagnosis and treatment of the infection and should always be performed on appropriate specimens. Numerous gram-positive cocci along with polymorphonuclear cells in purulent exudates, joint fluids, aspirated secretions, and other body fluids, are easily seen when these sites are infected with staphylococci. A culture should be done

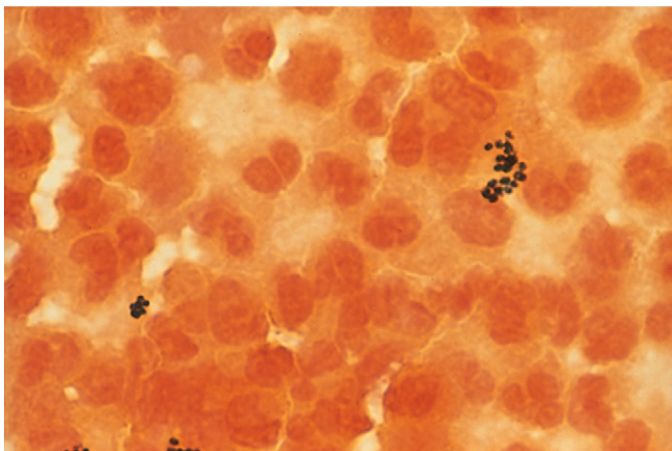


Fig. 14.2 Numerous gram-positive cocci in clusters, with many polymorphonuclear cells from an aspirated abscess in staphylococcal disease (Gram stain, original magnification $\times 1000$).

regardless of the results of the microscopic examination because the genus or species cannot be appropriately identified by microscopic morphology alone (Fig. 14.3). An aspirate is the best sample, whereas a single swab would be less satisfactory for both culture and smear results.

Isolation and identification

Staphylococci grow easily on routine laboratory culture media, particularly sheep blood agar (SBA). A selective medium such as mannitol salt agar (MSA), Columbia colistin–nalidixic acid agar (CNA), or phenylethyl alcohol (PEA) agar can be used for heavily contaminated specimens. The high NaCl concentration (7.5%) in MSA makes this medium selective for *Staphylococcus*, whereas the incorporation of mannitol and phenol red distinguishes *S. aureus* from most CoNS. CHROMagar Staph aureus (Becton Dickinson, Franklin Lakes, NJ) is a proprietary selective and differential medium for isolation and identification of *S. aureus*. CHROMagar MRSA can further classify *S. aureus* into MRSA or *methicillin-susceptible Staphylococcus aureus* (MSSA) strains with a higher level of sensitivity than traditional oxacillin screening methods.

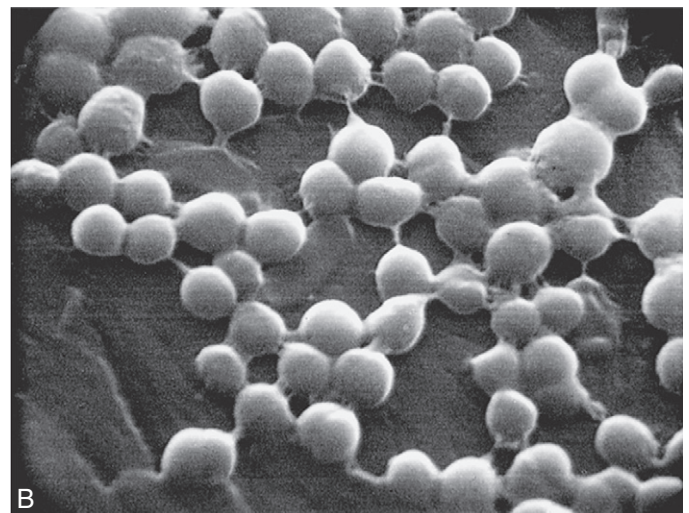
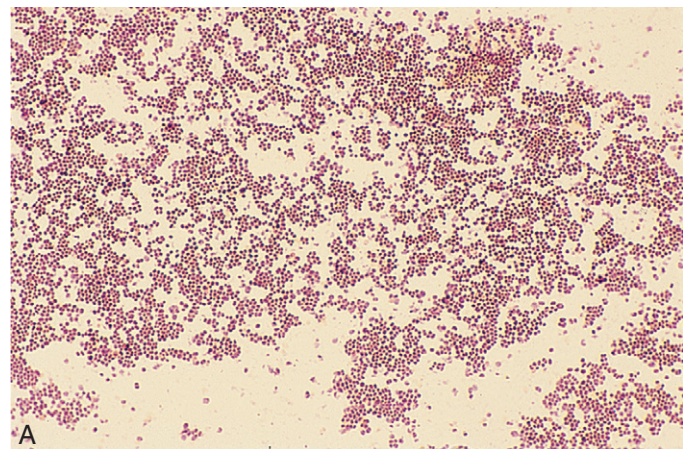


Fig. 14.3 **A**, Microscopic morphology of *Staphylococcus* spp. On Gram stain, gram-negative-looking cells show how older cells decolorize ($\times 1000$). **B**, Scanning electron micrograph showing the typical “clusters” of staphylococci ($\times 20,000$).

Cultural characteristics

Staphylococci produce round, smooth, white, creamy colonies on SBA after 18 to 24 hours of incubation at 35° to 37° C. *S. aureus* can produce hemolytic zones around the colonies (Fig. 14.4) and may rarely exhibit pigment production (yellow) with extended incubation. The SCVs grow as non-pigmented, nonhemolytic pinpoint-size colonies mixed with colonies exhibiting the normal phenotype.

S. epidermidis colonies are usually small- to medium-size, nonhemolytic, gray-to-white colonies (Fig. 14.5). Some can be weakly hemolytic. *S. saprophyticus* forms slightly larger colonies, with about 50% of the strains producing a yellow pigment. *S. haemolyticus* produces medium-size colonies, with moderate or weak hemolysis and variable pigment production. Colonies of *S. lugdunensis* are often hemolytic and medium size, although small colony variants can occur. Identification of staphylococci on the basis of colony morphology alone should not be done.

Identification methods

Staphylococci have been traditionally differentiated from micrococci on the basis of oxidation-fermentation (O/F)

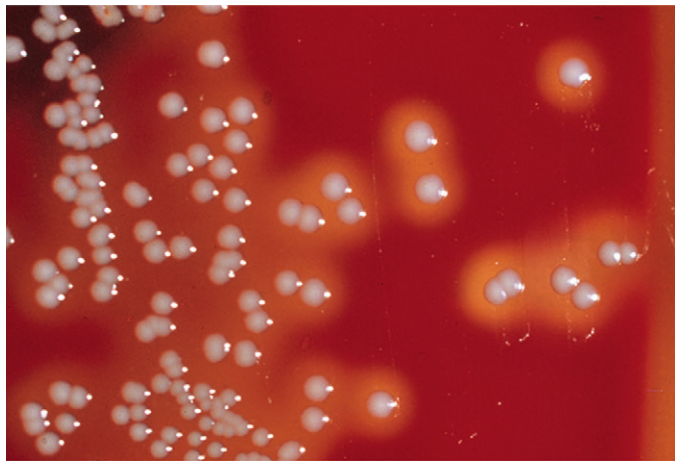


Fig. 14.4 *Staphylococcus aureus* growing on sheep blood agar showing β -hemolytic, creamy, buttery-looking colonies.

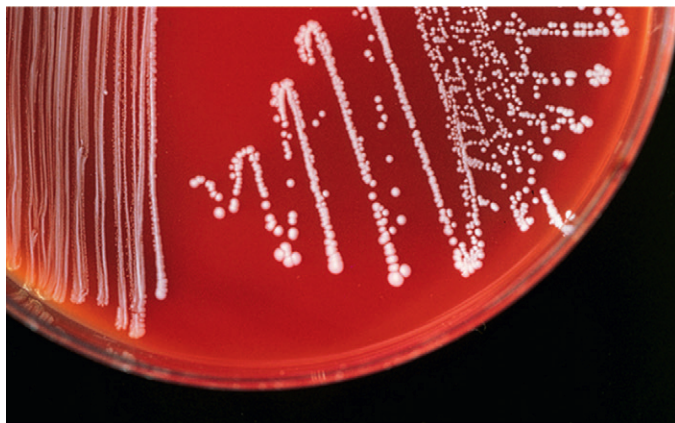


Fig. 14.5 Coagulase-negative staphylococci growing on sheep blood agar revealing nonhemolytic, white, creamy colonies.

reactions produced in O/F glucose medium. Staphylococci ferment glucose, whereas micrococci fail to produce acid under anaerobic conditions. However, the O/F tests do not sufficiently discern certain weak acid producers, such as *Kocuria kristinae* (formerly *Micrococcus kristinae*), and staphylococci that fail to grow or produce acid anaerobically, such as *S. saprophyticus*, *S. auricularis*, *S. hominis*, *S. xylosus*, and *S. cohnii*. Tests to differentiate micrococci from staphylococci are shown in Table 14.1. A modified oxidase test (see procedure in Appendix D on Evolve) such as the Remel Microdase Disc (Thermo Fisher Scientific, Waltham, MA) can be used to rapidly differentiate staphylococci from micrococci. Most staphylococci test negative, whereas micrococci test positive. Table 14.4 outlines key characteristics for differentiating staphylococci from other gram-positive cocci. Many commercial multitest systems have incorporated these traditional biochemical tests. In addition, molecular testing, plasmid typing, and fatty acid analysis and more recently MALDI-TOF mass spectrometry have been used for species and strain identification.

S. aureus is often identified by coagulase tests. Clumping factor, formerly referred to as *cell-bound coagulase*, causes agglutination in human, rabbit, or pig plasma. Clumping factor on the surface of the bacterial cells directly converts fibrinogen to fibrin, which precipitates onto the cell surface, causing agglutination, and this was performed on a glass slide using a heavy suspension of organism mixed with saline and a drop of plasma. A negative result was followed up by a tube coagulase test, which is described later. Because of the insensitivity of this slide test, only the tube coagulase or agglutination tests should be used.

The tube coagulase method (see Appendix D on Evolve for procedure) detects staphylocoagulase, or free coagulase. Staphylocoagulase is an extracellular molecule that causes a clot to form when bacterial cells are incubated with plasma (Fig. 14.6). Staphylocoagulase reacts with a thermostable, thrombin-like molecule called *coagulase-reacting factor* (CRF) to form coagulase-CRF complex. The coagulase-CRF complex resembles thrombin and indirectly converts fibrinogen to fibrin. The clot formed in the tube may undergo autolysis (caused by fibrinolysin), giving the appearance of a negative result. The medical laboratory scientist should look for clot formation after 4 hours of incubation at 37° C. If no clot appears, the tube should be left at room temperature and checked the next day. Fibrinolysin activity is enhanced at 37° C. Table 14.5 lists coagulase-positive staphylococci and identifies their clinical source and significance. Medical laboratory scientists must be aware that staphylococci other than *S. aureus* produce clumping factor or staphylocoagulase (Table 14.6).

Testing for *pyrrolidonyl arylamidase activity* (PYR) (see Appendix D on Evolve for procedure) can be used to differentiate *S. aureus* (negative) from *S. lugdunensis*, *S. intermedius*, and *S. schleiferi* (positive). The substrate *pyroglutamyl- β -naphthylamide* (L-pyrrolidonyl- β -naphthylamide) is hydrolyzed to L-pyrrolidone and β -naphthylamine, which combines with *p*-dimethylaminocinnamaldehyde to form a red compound. Some laboratory scientists obtain more reliable results differentiating *S. aureus* (positive) from *S. intermedius* (negative) with the Voges-Proskauer (VP) test. In the VP test, a positive result is the formation of acetoin from glucose or pyruvate. *S. intermedius* is an animal pathogen, and most human infections are associated with animal bites. *S. lugdunensis*, *S. haemolyticus*, and *S. schleiferi* are also VP test positive.

Table 14.4 Differentiation among staphylococci and other gram-positive cocci

Characteristic	Staphylococci	Enterococci	Streptococci	Aerococci	Alloiococci	Planococci	Macrococci	Micrococci	<i>Rothia mucilaginosa</i>
Strict anaerobe	—	—	d	—	—	—	—	—	—
Facultative anaerobe	d	+	+	+	—	—	±	—	+
Motility	—	d	—	—	—	+	—	—	—
Growth on NaCl agar									
5% NaCl	+	+	d	+	+	+	+	+	—
6.5% NaCl	+	+	d	+	+	+	+	+	—
12% NaCl	d	(±)	—	+	ND	+	+/_	d	+
Catalase	+	—	—	—	±	+	+	+	±
Benzidine test	+	—	—	—	—	+	+	+	+
Anaerobic acid from glucose	d	+	+	(+)	ND	—	—	—	+
Lysostaphin (200 mg/mL)	—	+	+	+	ND	+	—	+	+
Erythromycin (0.4 µg/mL)	+	+	—	ND	ND	ND	+	— ^a	ND
Bacitracin (0.04-unit disk)	+	+	d	—	ND	ND	+	—	—

^aA few *Micrococcus* strains demonstrate high-level (minimal inhibitory concentration ≥ 50 µg/mL) erythromycin resistance.

+, $\geq 90\%$ of species or strains positive; ±, $\geq 90\%$ of species or strains weakly positive; —, $\geq 90\%$ of species or strains negative; d, 11% to 89% of species or strains positive; /, delayed reaction; ND, not determined.

From Becker, K., et al. (2019). *Staphylococcus*, *Micrococcus*, and other catalase-positive cocci. In K. C. Carroll, et al. (Eds.). *Manual of clinical microbiology* (12th ed., p.370). Washington, DC: ASM Press.

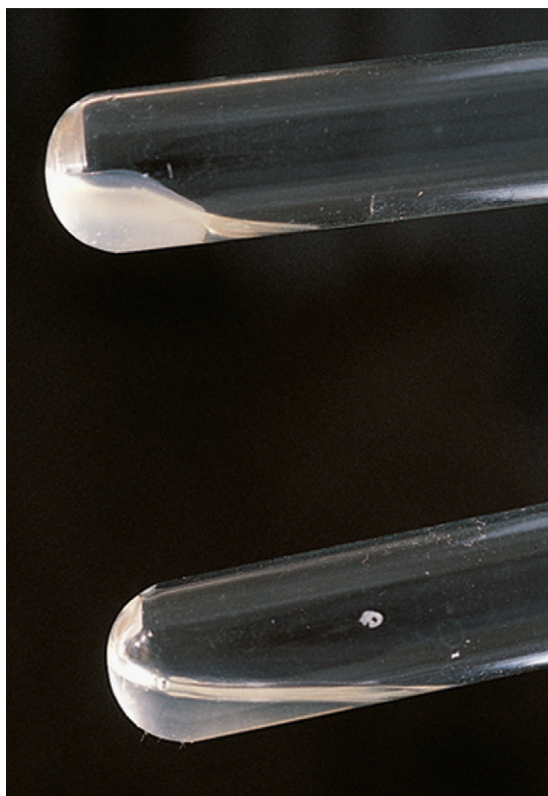


Fig. 14.6 Tube coagulase test detects extracellular enzyme “free coagulase.” The top tube is coagulase positive.

Table 14.5 Coagulase-positive staphylococci and their clinical source and significance

Staphylococcus species	Source
<i>S. aureus</i>	Human ^a , animal
<i>S. aureus</i> subsp. <i>anaerobius</i>	Animal ^a
<i>S. hyicus</i>	Animal ^a
<i>S. agnetis</i>	Animal
<i>S. intermedius</i>	Animal ^a
<i>S. pseudintermedius</i>	Animal ^a , human
<i>S. schleiferi</i> subsp. <i>coagulans</i>	Animal ^a
<i>S. delphini</i>	Animal
<i>S. lutrae</i>	Animal

^aCommon in human or veterinary disease.

Isolates that do not produce **staphylocoagulase** are reported as CoNS. Urine isolates that are coagulase negative are tested further to identify *S. saprophyticus*. Presumptive identification of *S. saprophyticus* is accomplished by testing for novobiocin susceptibility (see [Appendix D](#) on Evolve for procedure) using a 5-μg novobiocin disk ([Fig. 14.7](#)). *S. saprophyticus* is resistant to novobiocin, whereas most other CoNS are susceptible. [Fig. 14.8](#) shows a schema for the identification of clinically significant staphylococci. [Table 14.6](#) outlines key tests for the identification of clinically significant *Staphylococcus* spp., including some CoNS.

Rapid methods of identification

Numerous commercial rapid agglutination test kits are available for differentiating *S. aureus* from CoNS. Among these are the BBL Staphyloslide (BD-BBL, Sparks, MD), Staphaurex (Thermo Fisher Scientific, Waltham, MA), and BACTiStaph (Thermo Fisher Scientific; [Fig. 14.9](#)). These kits use plasma-coated carrier particles, such as latex. The plasma detects both clumping factor (with fibrinogen) and protein A in the cell wall of *S. aureus* (with IgG). These kits often have a higher specificity and sensitivity than the traditional plasma slide test and are quicker than the tube coagulase test. Therefore they are commonly used in clinical laboratories. They are particularly useful for the identification of MRSA organisms, which are often weakly positive or negative in the slide coagulase test. Negative results should be confirmed with the tube coagulase test, nucleic acid amplification test, or MALDI-TOF mass spectrometry.

Third-generation agglutination kits, besides detecting protein A and clumping factor, contain antibodies that bind capsular antigens 5 and 8, or other surface molecules. Whereas these assays are more sensitive, they are generally less specific. False-positive results can occur with some CoNS, including *S. saprophyticus*, *S. hominis*, and *S. haemolyticus*. Careful consideration of source, colony morphology, and susceptibility pattern can eliminate some errors.

Although numerous automated and rapid multitest biochemical systems for the identification of staphylococci are available, their accuracy differs (70% to 90%). Most systems are able to identify *S. aureus*, most *S. epidermidis*, and *S. saprophyticus* strains accurately as well as some other staphylococcal species, including *S. capitis*, *S. haemolyticus*, *S. simulans*, *S. lugdunensis*, and *S. intermedius*.

Molecular methods and other rapid identification systems have been introduced into the clinical setting. Real-time polymerase chain reaction (PCR) is available for identifying both MRSA and MSSA. It has been used as an adjunct to infection control practices to reduce MRSA infections, especially in select targeted populations that would benefit from rapid testing results, such as patients in intensive care units and other select patient populations. Molecular methods are able to directly identify staphylococci from a positive blood culture sample as an aid in the diagnosis of sepsis and for targeted antimicrobial therapy treatment. Several commercial platforms, including the FilmArray BCID panel (BioFire, Durham, NC) based on multiplex PCR and the Verigene blood culture assay (Nanosphere, Northbrook, IL), which uses a proprietary chemistry method to detect nucleic acids and proteins, are available. These systems provide detection of *Staphylococcus*, *S. aureus*, and the *mecA* gene for methicillin resistance detection within 1 to 2 hours after a positive blood culture bottle has been identified.

Newer technologies, including non-PCR-based molecular techniques, are currently being evaluated, including the T2 Biosystems (Lexington, MA) and Qvella (Richmond Hill, ON) technologies for direct detection from patient blood samples, without the need of the 24- to 48-hour growth incubation phase. The major advantage of these prototype technologies is the decreased time for administration of appropriate antimicrobial therapy and likely reduced mortality and morbidity. One major disadvantage, however, is that the clinical laboratory must be able to grow the organism if antimicrobial susceptibilities are needed and, therefore, cannot rely solely

Table 14.6 Key tests for identification of the most clinically significant *staphylococcus* species

Test	<i>S. aureus</i>	<i>S. epidermidis</i>	<i>S. haemolyticus</i>	<i>S. lugdunensis</i>	<i>S. saprophyticus</i> subsp. <i>saprophyticus</i>	<i>S. schleiferi</i> subsp. <i>schleiferi</i>	<i>S. simulans</i>
Colony pigment	+	–	d	d	d	–	–
Staphylocoagulase	+	–	–	–	–	–	–
Clumping factor	+	–	–	(+)	–	+	–
Heat-stable nuclease	+	–	–	–	–	+	–
Alkaline phosphatase	+	+ ^a	–	–	–	+	(d)
Pyrrolidonyl arylamidase	–	–	+	+	–	+	+
Ornithine decarboxylase	–	(d)	–	+	–	–	–
Urease	d	+	–	d	+	–	+
β-Galactosidase	–	–	–	–	+	(+)	+
Acetoin production	+	+	+	+	+	+	d
Novobiocin resistance	S	S	S	S	R	S	S
Polymyxin B resistance	R	R	S	d	S	S	S
Acid (aerobically from)							
D-Trehalose	+	–	+	+	+	d	d
D-Mannitol	+	–	d	–	d	–	+
D-Mannose	+	(+)	+	+	–	+	d
D-Turanose	+	(d)	(d)	(d)	+	–	–
D-Xylose	–	–	–	–	–	–	–
D-Cellobiose	–	–	–	–	–	–	–
Maltose	+	+	+	+	+	–	(±)
Sucrose	+	+	+	+	+	–	+

^aA low but significant number (6% to 15%) of clinical isolates are alkaline phosphatase negative.

+, ≥90% of strains positive; ±, ≥90% of strains weakly positive; –, ≥90% of strains negative; d, 11% to 89% of strains positive; (d), delayed reaction; R, resistant; S, susceptible.

Modified from Bannerman, T. L. (2007). *Staphylococcus, micrococcus, and other catalase: positive cocci that grow aerobically*. In P. R. Murray, et al. (Eds.), *Manual of clinical microbiology* (9th ed.). Washington, DC: ASM Press.

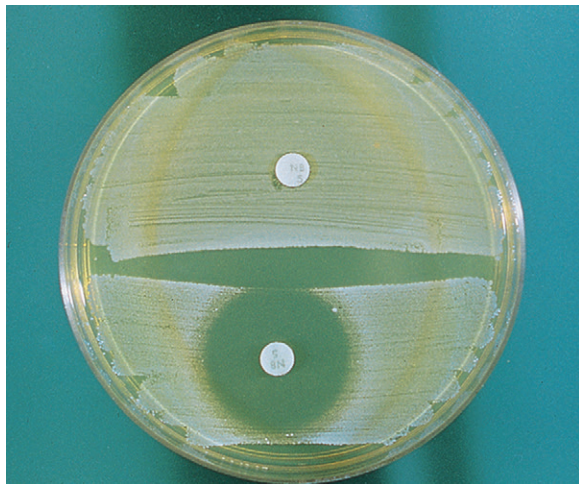


Fig. 14.7 Novobiocin susceptibility test to differentiate coagulase-negative staphylococci isolated from urine samples. *Staphylococcus saprophyticus* (top) is resistant to novobiocin, indicated by the lack of a zone of inhibition around the disk.

on molecular methods. Also, in the case of mixed infection, molecular methods may identify only one of the organisms present, making it essential to keep traditional methods a common practice in most cases. In comparing molecular

assays with conventional methods and the need to perform backup conventional methods, the laboratory must determine the requirements in the patient population along with the test's sensitivity, specificity, positive predictive value, and negative predictive value.

MALDI-TOF mass spectrometry continues to gain acceptance in clinical microbiology laboratories (see [Chapter 11](#)). Technologic advances, including the use of more molecular and mass spectrometry methods, are expected to increase the ability to identify staphylococci and other clinically relevant bacteria accurately and rapidly. The organism must be in a pure isolated colony and grown for a specified duration, at a specific incubation temperature, and on certain growth media. MALDI-TOF mass spectrometry cannot be used to distinguish genetic differences, such as MRSA versus MSSA strains.

Antimicrobial susceptibility

Routine testing of staphylococcal isolates can be easily performed in the laboratory using standard guidelines issued by the Clinical and Laboratory Standards Institute (CLSI). When using commercial tests, laboratories must adhere to the manufacturers' recommended procedures. Antimicrobial

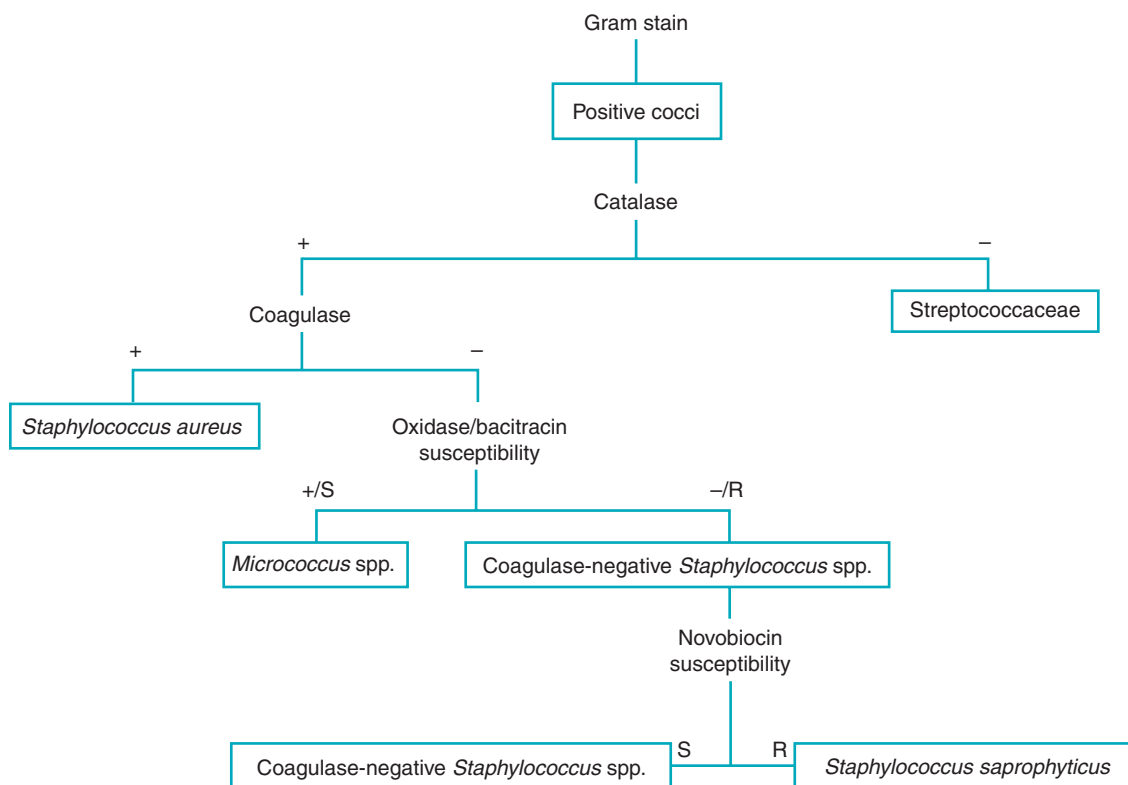


Fig. 14.8 Schema for the identification of staphylococcal species. *Note:* Other *Staphylococcus* spp. that are coagulase positive besides *S. aureus* include *S. schleiferi* and *S. lugdunensis* (which can be slide test positive), *S. intermedius*, *S. pseudintermedius*, and *S. hyicus* (tube positive and slide test positive). R, Resistant; S, susceptible.



Fig. 14.9 Slide coagulase test (Remel BACTiStaph), a latex agglutination method commercially available for the detection of both clumping factor and protein A. (Courtesy Thermo Fisher Scientific, Waltham, MA.)

susceptibility testing of CoNS depends on the source and determination if the isolate is a contaminant or a likely pathogen. The latest interpretive standards for guidance and interpretation must be used when testing CoNS. Serious infections with *S. aureus* and *S. lugdunensis* require susceptibility testing. Because of the production of β -lactamases (penicillinases), which break down the β -lactam ring of many penicillins, most *S. aureus* isolates are resistant to penicillin. Increasing resistance to alternative antimicrobial agents is a major concern.

Methicillin-resistant staphylococci

Penicillin-resistant strains require treatment with penicillinase-resistant penicillins, such as nafcillin or oxacillin. Although methicillin is no longer used in the United States, isolates that are resistant to nafcillin or oxacillin have been traditionally termed *methicillin-resistant staphylococci*, for example, MRSA and **methicillin-resistant *Staphylococcus epidermidis*** (MRSE). Whereas MRSA has remained a serious health concern, some studies have shown a decrease in deaths in health care settings caused by invasive MRSA infections. This may be a result of more stringent infection control measures. However, since the 1990s, the incidence of **community-associated methicillin-resistant *Staphylococcus aureus*** (CA-MRSA) infections has increased (>50% of *S. aureus* isolates in some areas of the United States), and these infections can be found in patients who lack traditional healthcare-associated risk factors, such as recent hospitalization, long-term care, hemodialysis, or indwelling devices. CA-MRSA infections and outbreaks have been reported among athletes, prison inmates, military recruits in close-contact environments, pediatric patients, and tattoo recipients. Although **hospital-associated methicillin-resistant *Staphylococcus aureus*** (HA-MRSA) rates have stabilized and even declined, vigilance will still be needed to ensure that CA-MRSA rates are monitored, patient populations are educated, and adequate control measures are applied. The Centers for Disease Control and Prevention (CDC) estimates that 72,444 total MRSA cases occur annually.

MRSA infections—whether HA-MRSA or CA-MRSA infections—are costly and pose a serious threat to health institutions. Control of MRSA requires strict adherence to infection control practices, including barrier protection, contact isolation, and handwashing compliance. The use of rapid tests for MRSA may aid in the control of this agent. Vancomycin remains the treatment of choice for MRSA infection, but concerns with resistance to glycopeptides call for restrictive use of these drugs and selective reporting by the laboratory.

Case check 14.2

Based on MALDI-TOF mass spectrometry, the patient in the Case in Point was diagnosed with a *S. pseudintermedius* wound infection. This bacterium produces a positive tube coagulase test result, which could cause it to be confused with *S. aureus*. However, *S. pseudintermedius* is negative for clumping factor and should be negative in agglutination assays. Isolates are typically oxacillin resistant.

In the past, oxacillin was generally used for detection of methicillin resistance for staphylococcal species. The latest CLSI M100 document recommends that cefoxitin be used to detect oxacillin (methicillin) resistance. Cefoxitin is a better inducer of *mecA*-mediated resistance. At a minimum, the laboratory should report susceptibilities for penicillin and cefoxitin and/or oxacillin. *S. lugdunensis* has been given the same minimal inhibitory concentration breakpoint for cefoxitin as *S. aureus*, which is different from other CoNS. Some non-*S. epidermidis* strains lack *mecA*, and testing for penicillin-binding protein 2a (PBP2a) or cefoxitin disk testing might be more appropriate. Medical laboratory scientists must always refer to the latest susceptibility guides for correct interpretations. MRSA isolates should be considered resistant to all β -lactam antibiotics, including the carbapenems, except for the fifth-generation cephalosporins with MRSA activity.

MRSA populations are often heterogeneous in resistance to β -lactams, meaning one subpopulation is susceptible, whereas another is resistant to methicillin. Even though nearly all cells possess the genetic information to be resistant, only a small fraction (1 in 10^8 to 10^4 cells) expresses the resistance phenotype. Growth of the resistant subpopulation is enhanced at a neutral pH, sodium chloride concentration of 2% to 4%, cooler incubation temperature (30° to 32° C), and prolonged incubation (up to 48 hours).

Media containing oxacillin, or preferably cefoxitin, can be used to screen for MRSA in clinical samples, such as nasal specimens. These media also can differentiate MRSA isolates from isolates that are hyperproducers of β -lactamase, or **borderline oxacillin-resistant *Staphylococcus aureus* (BORSA)** strains, which would not grow on these plates. Chromogenic selective differential media, such as MRSA Select (Bio-Rad Laboratories, Hercules, CA), Spectra MRSA (Thermo Fisher Scientific), and CHROMagar MRSA (BD-BBL), have the ability to identify MRSA directly from clinical samples. High sodium chloride concentration and antimicrobial compounds such as cefoxitin are incorporated in the media and inhibit non-staphylococci and non-MRSA isolates. After 24 or 48 hours of incubation, MRSA isolates produce a colored colony, whereas MSSA and most other organisms are inhibited or produce a noncolored colony. Automated antimicrobial

susceptibility systems using cefoxitin are also generally able to identify MRSA.

Most oxacillin resistance is caused by the *mecA* gene, which is carried on a mobile cassette known as SCC_{mec}. The gene codes for an altered **penicillin-binding protein (PBP)**, PBP2a, also designated *PBP2*. The altered PBP does not bind oxacillin, rendering the drug ineffective. Latex agglutination tests are available to detect these altered PBPs, and they provide an alternative method for testing and confirmation of oxacillin resistance. This test can be performed on both CoNS and *S. aureus*.

The gold standard for MRSA detection is the detection of the *mecA* gene by using nucleic acid probes or PCR amplification. Many systems are available for direct detection from anterior nares swabs, such as the BD GeneOhm MRSA assay and the Xpert MRSA assay (Cepheid, Sunnyvale, CA) using the GeneXpert system, both of which use real-time PCR and produce results within several hours. Numerous molecular systems can identify both MRSA and MSSA in the same assay. Either type of test, with a decreased time to detection, has the potential to reduce transmission of both HA-MRSA and CA-MRSA within the hospital setting. These assays are moderate or high-complexity tests at the moment, but Clinical Laboratory Improvement Amendments–waived versions may become available as point-of-care testing in the future and could be used in nursing homes and long-term health care facilities. In addition, some PCR assays are able to identify the most common CA-MRSA strains, which are associated with a high mortality and morbidity rate and often carry the *PVL* genes. More recently, scientists in Europe have identified a new type of SCC_{mec} from a different lineage of the previously described gene. This new variant is not detected by conventional and real-time PCR assays.

CA-MRSA can cause healthcare-associated infections, and this might have an important implication in treatment and epidemiology. The Joint Commission, an accreditation agency for health care facilities, established safety goals for a laboratory-based alert system to identify MRSA, and many states already have either enacted or pending legislation requiring MRSA screening and reporting.

Vancomycin-resistant staphylococci

Vancomycin is the drug of choice and sometimes the only drug available for serious staphylococcal infections, and the development of vancomycin resistance is a serious concern for the medical community. In 1996, the first **vancomycin-intermediate *Staphylococcus aureus* (VISA)** strains were recovered in Japan. Automated antimicrobial susceptibility testing methods may be unreliable in detecting these isolates. The disk diffusion procedure also has limitations in detecting resistance. It has been suggested that clinical microbiology laboratories use more than one method to detect VISA. Because of the difficulty in identifying VISA, their incidence might be underreported. In 2002, isolates of true **vancomycin-resistant *Staphylococcus aureus* (VRSA)** were reported in the United States, isolated from patients undergoing long-term vancomycin treatment. So far, most of the isolates recovered in the United States have been from patients with underlying conditions. Detection of these isolates should be confirmed by a reference method, and reporting should follow CDC guidelines. Adherence to infection control practices



Fig. 14.10 D-zone test–positive isolate showing flattening of the clindamycin (CC) zone adjacent to the erythromycin (E) disk and the characteristic D-like pattern.

and CDC guidelines for vancomycin resistance may limit the emergence of this highly resistant organism.

Macrolide resistance

Resistance to other categories of antimicrobials such as macrolides might not always be readily apparent by routine testing. Clindamycin, a macrolide, is frequently used in staphylococcal skin infections; additional testing using a modified double-disk diffusion test (**D-zone test**) might be useful when discrepant macrolide test results are obtained (e.g., erythromycin resistant and clindamycin susceptible). Erythromycin and clindamycin susceptibility results are normally the same. However, staphylococcal resistance to clindamycin is occasionally inducible, meaning it is detectable in vitro only when the bacteria are also exposed to erythromycin.

Inducible clindamycin resistance can be detected by disk diffusion by placing an erythromycin disk near a clindamycin disk and using the latest performance standards for susceptibility testing. If an isolate possesses inducible clindamycin resistance, the bacteria grow around the erythromycin disk and in the area of the agar where the two drugs overlap. However, a zone of inhibition is observed on the side of the clindamycin disk farther away from the erythromycin disk, flattening the clindamycin zone, which looks like the letter *D* (Fig. 14.10). Some automated methods have the ability to detect this resistance. It is important for laboratories to keep up with the latest trends in antimicrobial resistance and to be aware of limitations that can occur with susceptibility testing.

POINTS TO REMEMBER

- The staphylococci are catalase-positive, gram-positive cocci.
- *Staphylococcus aureus* is the primary pathogen within this genus, and the isolation of *S. aureus* from any source should be considered clinically significant.
- *S. aureus* produces many virulence factors, including protein A, enterotoxins, toxic shock syndrome toxin-1, exfoliative toxin, cytolytic toxins, and numerous exoenzymes.

- *S. aureus* is associated with numerous diseases, including skin infections, scalded skin syndrome, toxic shock syndrome, food poisonings, bacteremia, osteomyelitis, and pneumonia.
- *Staphylococcus epidermidis* and other coagulase-negative staphylococci (CoNS) have been linked to important hospital-associated infections, often associated with foreign body implants.
- The pathogenicity of *S. lugdunensis*, a CoNS, is more like *S. aureus* than the other CoNS.
- CoNS recovered from sterile sites and sites associated with indwelling devices should be considered potential pathogens.
- HA-MRSA and CA-MRSA are important and costly health care concerns.
- *Staphylococcus saprophyticus* is an important cause of urinary tract infections, especially in younger women. The identification of *S. saprophyticus* from urine specimens should be done, especially if the bacteria are predominant, because even lower numbers can be significant.
- *S. aureus* is frequently separated from less pathogenic species by being tube coagulase positive.
- *S. saprophyticus* is resistant to novobiocin, whereas many other CoNS are sensitive.
- Increasing antimicrobial resistance is a problem with the staphylococci, particularly *S. aureus*.

LEARNING ASSESSMENT QUESTIONS

1. Which types of infections are associated with *S. aureus*?
2. In which populations are *S. aureus* infections most likely to occur?
3. How does protein A contribute to the virulence of *S. aureus*?
4. Which toxin causes toxic shock syndrome?
5. Which type of toxin is associated with staphylococcal scalded skin syndrome?
6. Which toxins are involved in staphylococcal food poisoning?
7. In which clinical condition would coagulase-negative staphylococci be significant, and in which condition might they be considered a contaminant?
8. Which coagulase-negative staphylococci are considered more significant and might need to be identified to the species level?
9. What are the two types of coagulase produced by *S. aureus*, and which one can be used as a confirmatory test for coagulase in a clinical laboratory?
10. How is *S. aureus* differentiated from similar isolates?
11. Which test is used to identify *S. saprophyticus*?
12. What is the significance of an oxacillin-resistant *S. aureus* isolate?
13. What are some risk factors associated with HA-MRSA and CA-MRSA?
14. What are the recommendations for detecting oxacillin, clindamycin, and vancomycin resistance?
15. What are the methods for identifying staphylococci?
16. Which rapid methods would be appropriate to identify a staphylococcal isolate, and when should they be used?
17. Which of the following *Staphylococcus* species is novobiocin resistant?
 - a. *S. epidermidis*
 - b. *S. saprophyticus*
 - c. *S. haemolyticus*
 - d. *S. aureus*

18. Infections following prosthetic heart valve insertion are noted in a hospital. Methicillin-resistant strains of which of the following species are associated with this condition?
 - a. *S. epidermidis*
 - b. *S. saprophyticus*
 - c. *S. haemolyticus*
 - d. *S. salivarius*
19. Culture from an infected lesion yields catalase-positive, gram-positive cocci. The colonies appear β -hemolytic, smooth, butyrous, and when tested, produce a positive tube coagulase test. Which of the following species is involved in this infection?
 - a. *S. epidermidis*
 - b. *S. saprophyticus*
 - c. *S. haemolyticus*
 - d. *S. aureus*
20. What gene encodes for oxacillin resistance in certain staphylococcal species?
 - a. *aac*
 - b. *Cat*
 - c. *mecA*
 - d. *vanA*

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Streptococcus, Enterococcus, and other catalase-negative, gram-positive cocci

Kalavati Suvana and Connie R. Mahon

CHAPTER OUTLINE

General characteristics, 325

- Cell wall structure, 325
- Hemolysis, 326

Clinically significant streptococci and Streptococcus-like organisms, 326

- Streptococcus pyogenes*, 327
- Streptococcus agalactiae*, 330
- Group C and G streptococci, 332
- Streptococcus pneumoniae*, 333
- Viridans streptococci, 334
- Enterococcus*, 336
- Streptococcus*-like organisms, 338

Laboratory diagnosis, 340

- Classification schemes, 340
- Noncultural identification, 344
- Susceptibility testing, 345

Bibliography, 346

OBJECTIVES

After reading and studying this chapter, you should be able to:

1. Differentiate the general characteristics and isolation of streptococci and similar organisms.
2. Describe the Lancefield classification of streptococci.
3. Apply the knowledge of hemolytic patterns on sheep blood agar in the identification of streptococcal isolates.
4. Contrast the significance of streptococci and similar organisms commonly isolated in the clinical laboratory—both organisms that occur as normal biota and organisms that are potential pathogens.
5. Compare the virulence factors associated with the Streptococcaceae.
6. Explain how infections caused by the Streptococcaceae and similar organisms are established.
7. Justify the screening of women who are pregnant for group B streptococci.

8. Describe the characteristic morphology of streptococci in direct smears and from culture.
9. Given the microscopic and colony morphology of an organism isolated from a clinical sample, determine the appropriate biochemical tests for presumptive identification of the organism.
10. State the principle and purpose of each differential test used in the identification of the Streptococcaceae and similar organisms.
11. Discuss the major serologic tests used to detect antibodies that are produced after recent streptococcal infections.
12. Develop an algorithm for the identification of *Streptococcus* and *Enterococcus* species.

KEY TERMS

Acute glomerulonephritis

α -Hemolysis

β -Hemolysis

Bile solubility

Christie, Atkins, Munch-Peterson (CAMP) test

Capnophilic

Cellulitis

Empyema

Erysipelas

Hippurate hydrolysis test

Hyaluronidase

Impetigo

Lancefield classification system

Leucine aminopeptidase (LAP)

M protein

Necrotizing fasciitis (NF)

Optochin test

Pharyngitis

Pyogenic streptococci

Pyrrolidonyl- α -naphthylamide (PYR) hydrolysis

Rheumatic fever

Scarlet fever

Streptococcal pyrogenic exotoxins

Streptolysin O (SLO)

Streptolysin S

Sydenham chorea

Voges-Proskauer (VP) test

Case in point

A 9-year-old male complained of fever and sore throat over a 3-day period. On examination by his physician, the patient's pharynx was red, and both tonsils were swollen. Pronounced cervical lymphadenopathy was present. A swab of the tonsillar area was taken and inoculated on a sheep blood agar (SBA)

plate. After 24 hours of incubation, small, shiny, translucent colonies showing β -hemolysis were noted.

Issues to consider

After reading the patient's case history, consider:

- The most likely causative agent based on the presenting symptoms and colony morphology of the isolate
- Key tests to be performed on the bacterial isolate for identification
- Nonculture assays available for rapid identification of the isolate

In the past few decades, deoxyribonucleic acid (DNA) homology and DNA sequencing studies have led to numerous taxonomic changes to the family Streptococcaceae. The enterococci, formerly known as group D streptococci, have been classified in their own genus, *Enterococcus*. Similarly, lactococci, previously classified as group N streptococci, now belong in the genus *Lactococcus*. Although traditional phenotypic characteristics such as hemolysis and Lancefield classification (antigen serogrouping) are still useful in presumptive identification, nucleic acid studies have provided more information on the genetic relationships among different phenotypes of the members of the family Streptococcaceae. The family Streptococcaceae now includes five genera: *Streptococcus*, *Floricoccus*, *Lactococcus*, *Lactovum*, and *Okadaella*. The family Enterococcaceae includes the genus *Enterococcus* along with the genera *Bavariicoccus*, *Catelicoccus*, *Melissococcus*, *Pilibacter*, *Tetragenococcus*, and *Vagococcus*. The family Aerococcaceae includes the genera *Aerococcus*, *Abiotrophia*, *Dolosicoccus*, *Eremococcus*, *Facklamia*, *Falseniella*, *Fundicoccus*, *Globicatella*, *Hutsoniella*, *Ignavigranum*, *Ruoffia*, *Suicoccus*, and *Vaginisenealia*. This chapter presents the role of *Streptococcus* spp., *Enterococcus* spp., and other catalase-negative, gram-positive cocci in human disease, characteristics of the members in each genus, and how isolates are identified in the clinical microbiology laboratory.

General characteristics

Streptococcus and *Enterococcus* spp. are catalase-negative, gram-positive cocci that are usually arranged in pairs or chains (Fig. 15.1). A negative catalase test result differentiates streptococci and enterococci from staphylococci. Weak false-positive catalase reactions can be seen when growth is taken from media containing blood because of the peroxidase activity of hemoglobin. Compared with the cells of other gram-positive cocci, those of enterococci and some streptococci appear more elongated than spherical. The streptococcal cells are more likely to appear in chains when grown in broth cultures.

Most members of the genera *Streptococcus* and *Enterococcus* behave like facultative anaerobes. Because they grow in the presence of oxygen but are unable to use oxygen for respiration, they are considered aerotolerant anaerobes. Carbohydrates are metabolized fermentatively with lactic acid as the major end product; gas is not produced. Some species are **capnophilic**, requiring an increased concentration of carbon dioxide (CO_2), whereas the growth of other species is stimulated by an increased concentration of CO_2 , but CO_2 is not required. Growth is poor on nutrient media such as trypticase soy agar. On media enriched with blood or serum, growth is more pronounced. The colonies are usually small and transparent.

Cell wall structure

Streptococci possess a typical gram-positive cell wall consisting of peptidoglycan and teichoic acid. Most streptococci, except for many of the viridans group, have a group or common C carbohydrate (polysaccharide) that can be used to serologically classify an isolate. This classification scheme was developed in the 1930s by Rebecca Lancefield. After first recognizing the antigen in β -hemolytic streptococci, Lancefield was able to divide the streptococci into serologic groups designated by letters. Organisms in group A possess the same antigenic C carbohydrate, organisms in group B have the

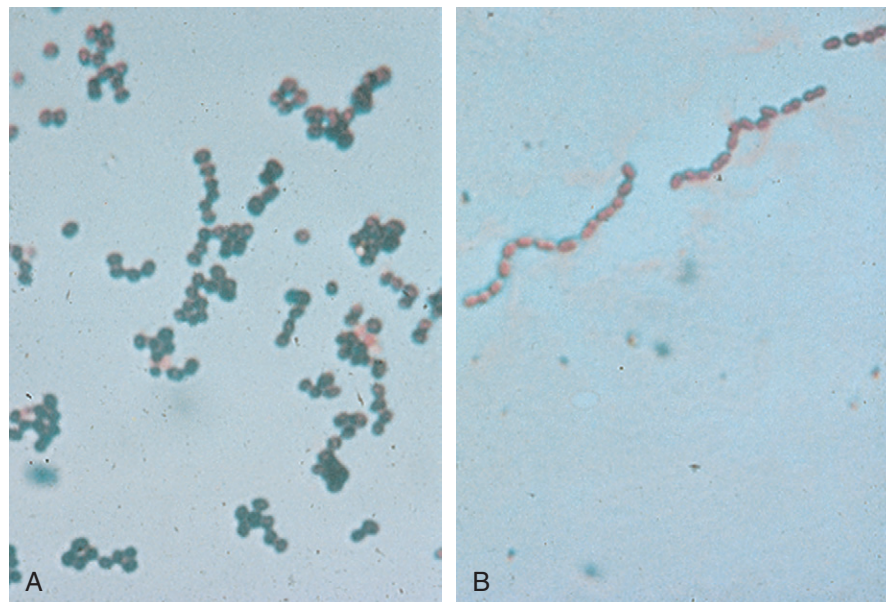


Fig. 15.1 Gram stain of *Streptococcus*. A, Solid medium. B, Liquid medium.

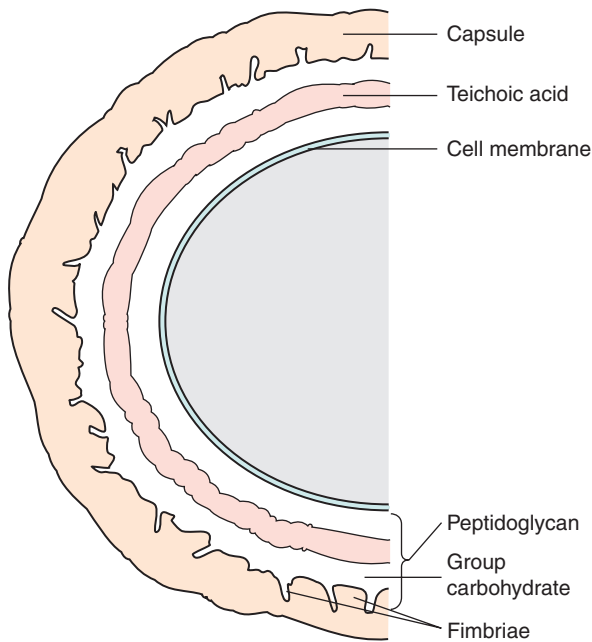


Fig. 15.2 The streptococcal cell wall.

Table 15.1 Types of Hemolysis

Hemolysis	Description
Alpha (α)	Partial lysis of RBCs around colony Greenish discoloration of area around colony
Beta (β)	Complete lysis of RBCs around colony Clear area around colony
Nonhemolytic	No lysis of RBCs around colony No change in agar
Alpha-prime (α') or wide zone	Small area of intact RBCs around colony surrounded by a wider zone of complete hemolysis

RBC, Red blood cell.

same C carbohydrate, and so on. A schematic diagram of the streptococcal cell wall is shown in Fig. 15.2. Some species can produce a type-specific polysaccharide capsule as well.

Hemolysis

The streptococci and similar organisms can produce numerous exotoxins that damage intact red blood cells (RBCs). The types of hemolysis on sheep RBCs are described in Table 15.1. When lysis of RBCs in the agar surrounding the colony is complete, the resulting area is clear; this is termed **β -hemolysis** (Fig. 15.3). Partial lysis of the RBCs results in a greenish discoloration of the area surrounding the colony and is termed **α -hemolysis** (Fig. 15.4).

When the RBCs immediately surrounding the colony are unaffected, the bacteria are described as *nonhemolytic*. Some older references term this result *γ -hemolysis*. However, because no lysis of the RBCs occurs, the term *γ -hemolysis* is confusing and is not recommended. Some isolates belonging to the viridans group produce what is called *wide-zone* or *α' hemolysis*. The

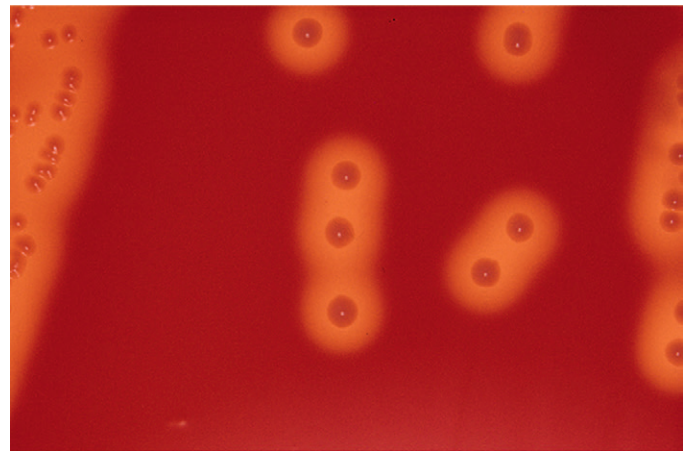


Fig. 15.3 β -Hemolytic streptococcal colonies on sheep blood agar.

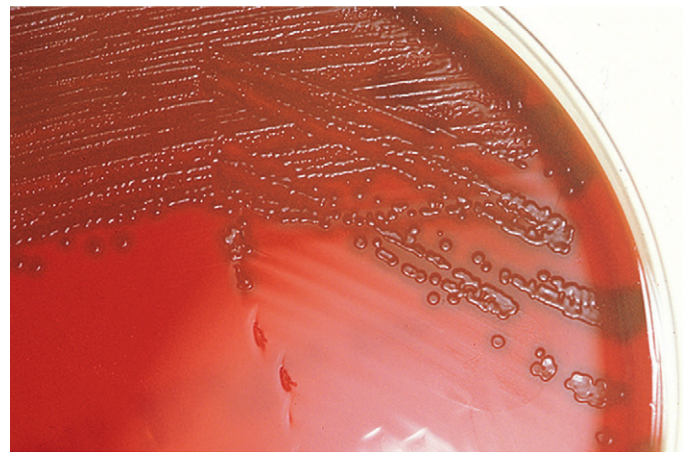


Fig. 15.4 α -Hemolytic streptococcal colonies on sheep blood agar.

colonies are surrounded by a very small zone of no hemolysis and then a wider zone of β -hemolysis. This reaction may be mistaken for β -hemolysis at first glance. The use of a dissecting microscope or handheld lens reveals the narrow zone of intact RBCs and the wider zone of complete hemolysis.

Case check 15.1

Organisms belonging to the family Streptococcaceae are catalase-negative, gram-positive cocci that are usually arranged in pairs or chains. The organisms are classified based on the hemolytic pattern on SBA and serologic grouping or typing of the C carbohydrate polysaccharide (i.e., Lancefield classification). The bacteria isolated from the patient in the Case in Point exhibited β -hemolysis on an SBA plate, and the identification was confirmed using a rapid serotyping assay.

Clinically significant streptococci and Streptococcus-like organisms

The role of streptococci and enterococci in disease has been known for more than 100 years. The range of infections caused by these organisms is wide and well-studied.

Table 15.2 Classification of *Streptococcus* and *Enterococcus*

Species	Lancefield Group Antigen	Hemolysis Types	Common Terms	Disease Association(s)
<i>S. pyogenes</i>	A	β^a	Group A streptococci	Rheumatic fever, scarlet fever, pharyngitis, glomerulonephritis, pyogenic infections
<i>S. agalactiae</i>	B	β^a	Group B streptococci	Neonatal sepsis, meningitis, puerperal fever, pyogenic infections
<i>S. dysgalactiae</i> , <i>S. equi</i>	C	β	Group C streptococci	Pharyngitis, impetigo, pyogenic infections
<i>S. bovis</i> group	D	α , none	Nonenterococcus member of viridans streptococci	Endocarditis, UTIs, nonpyogenic infections
<i>E. faecalis</i> , <i>E. faecium</i>	D	α , β , none	Enterococci	UTIs, pyogenic infections
<i>S. pneumoniae</i>	—	α	Pneumococcus	Pneumonia, meningitis, pyogenic infections
<i>Anginosus</i> group, <i>mutans</i> group, <i>mitis</i> group, <i>salivarius</i> group	A, C, F, G, N, or —	β , α , none	Viridans streptococci ^b	Usually, nonpyogenic infections, endocarditis, dental caries, abscesses in various tissues

^aOccasionally, isolates are found that are nonhemolytic.

^bAll four groups and the *S. bovis* group are referred to as viridans streptococci.

UTI, Urinary tract infection.

Some clinically important species and the diseases that they cause are listed in Table 15.2. Clinically isolated streptococci have historically been separated into β -hemolytic streptococci or **pyogenic** (pus-forming) **streptococci** and species that are non- β -hemolytic (nonpyogenic). Use of the term *pyogenic streptococci* is more precise because the group contains some species that are not β -hemolytic, and some β -hemolytic streptococci are not part of the pyogenic group. Pyogenic streptococci isolated frequently from humans include *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae* subsp. *equisimilis*, *Streptococcus dysgalactiae* subsp. *dysgalactiae*, and *Streptococcus anginosus* group (some species are α -hemolytic or nonhemolytic).

Streptococcus pyogenes

Antigenic structure

S. pyogenes has a cell wall structure similar to that of other streptococci and gram-positive bacteria. The group antigen is unique, placing the organism in Lancefield **group A**. **M protein** is attached to the peptidoglycan of the cell wall and extends to the cell surface. The M protein is essential for virulence.

Virulence factors

The best-defined virulence factor in *S. pyogenes* is M protein, encoded by the *emm* gene. The M-protein molecule causes the streptococcal cell to **resist phagocytosis** and plays a role in adherence of the bacterial cell to mucosal cells. Like staphylococcal protein A, M-protein can bind the fragment crystallizable (Fc) of human antibody; however, this effect on virulence is unknown. More than 200 different serotypes and subtypes of M protein exist, identified as M1 (*emm1*), M2 (*emm2*), etc. Resistance to infection with *S. pyogenes* appears to be related to the presence of type-specific antibodies to the M protein.

This means that an individual with antibodies against M5 is protected from infection by *S. pyogenes* with the M5 protein but remains unprotected against infection with the roughly 200 remaining M-protein serotypes. **The M1 serotype is the most common serotype seen in pharyngitis.** More recently, microarray analyses of group A streptococci (GAS) serotype M1 isolates have been useful in identifying the genes associated with pharyngitis versus invasive disease. M-like proteins, encoded by the *Enn* and *Mrp* genes, are additional surface proteins with similar functions as M protein. M and M-like proteins bind the **Fc portion of immunoglobulin G, immunoglobulin A, or both.** A study of 1,688 global isolates of group A streptococci found that 85% encoded both *Enn* and *Mrp* proteins.

Additional virulence factors associated with **GAS include fibronectin-binding protein (protein F), lipoteichoic acid, hyaluronic acid capsule, and extracellular products including hemolysins, toxins, and enzymes.** Lipoteichoic acid and protein F are adhesion molecules that mediate attachment to host epithelial cells. Lipoteichoic acid, which is affixed to proteins on the bacterial surface, in concert with M proteins and fibronectin-binding protein, secures the attachment of streptococci to the oral mucosal cells. The hyaluronic acid capsule of *S. pyogenes* is weakly immunogenic. **The capsule prevents opsonized phagocytosis by neutrophils or macrophages.** The capsule also allows the bacterium to mask its antigens and remain unrecognized by its host's immune system.

Other **products** produced by *S. pyogenes* include **streptolysin O (SLO)**, streptolysin S, deoxyribonuclease (DNase), streptokinase, hyaluronidase, and erythrogenic exotoxins capable of producing an **erythematous sandpaper-like rash.** Although all of these products have been postulated to play a role in virulence, the exact role each has in infection is unclear. *S. pyogenes* **secretes four different DNases:** A, B, C, and D. All strains produce at least one DNase; the most common is DNase B. These enzymes are antigenic, and antibodies to DNase can be detected after infection.

SLO is responsible for hemolysis on SBA plates incubated anaerobically. The O refers to this hemolysin being oxygen labile. It is active only in the reduced form, which is achieved in an anaerobic environment. SLO lyses leukocytes, platelets, and other cells as well as RBCs. SLO is highly immunogenic, and infected individuals readily form antibodies to the hemolysin. These antibodies can be measured in the antistreptolysin O (ASO) test to determine whether an individual has had a recent infection with *S. pyogenes*. **Streptolysin S** is oxygen stable, lyses leukocytes, and is nonimmunogenic. The hemolysis seen around colonies incubated aerobically is caused by streptolysin S.

GAS cause the lysis of fibrin clots through the action of streptokinase on plasminogen. The plasminogen is converted into a protease (plasmin), which lyses the fibrin clot. Antibodies to streptokinase can be detected after infection but are not specific indicators of GAS infection because group C and G streptococci also form streptokinase. **Hyaluronidase**, or spreading factor, is an enzyme that solubilizes the ground substance of mammalian connective tissues (hyaluronic acid). It was postulated that the bacteria use this enzyme to separate the tissue and spread the infection; however, no evidence to support this hypothesis exists.

Some strains of *S. pyogenes* cause a red spreading rash, referred to as scarlet fever, caused by streptococcal pyrogenic exotoxins, formerly called erythrogenic toxins. The four immunologically distinct exotoxin types found in *S. pyogenes* are SpeA, SpeB, SpeC, and SpeF. These toxins function as superantigens. Streptococcal superantigens belong to a family of highly mitogenic proteins secreted individually or in combinations by many *S. pyogenes* strains. These proteins share the ability to stimulate T-lymphocyte proliferation by interaction with class II major histocompatibility complex (MHC) molecules on antigen-presenting cells and specific variable β chains of the T-cell receptor. This interaction results in the production of interleukin-1, tumor necrosis factor, and other cytokines that appear to mediate the disease processes associated with these toxins.

Clinical infections

S. pyogenes colonizes the throat and skin on humans, making these sites the primary sources of transmission. Infections resulting from *S. pyogenes* include pharyngitis, scarlet fever, skin or pyoderma infections, and other septic infections. In addition, the sequelae rheumatic fever and acute glomerulonephritis can occur as a result of infection with *S. pyogenes*.

Bacterial pharyngitis

The most common clinical manifestations of GAS infection are pharyngitis and tonsillitis. Most cases of bacterial pharyngitis are caused by *S. pyogenes*. Streptococci from other groups, particularly C and G, have the capability to produce significant acute pharyngitis but are less commonly seen.

Streptococcal pharyngitis or "strep throat" is most often seen in children between 5 and 15 years of age. After an incubation period of 1 to 4 days, an abrupt onset of illness ensues, with sore throat, malaise, fever, and headache. Nausea, vomiting, and abdominal pain are not unusual. The tonsils and pharynx are inflamed. The cervical lymph nodes are swollen and tender. The disease ranges in intensity, and these symptoms may not be seen. It is not unusual to isolate a nearly

pure culture of *S. pyogenes* from the throat of a child with fever and complaint of only a mild sore throat. The symptoms subside within 3 to 5 days unless complications, such as peritonsillar abscesses, occur. The disease is spread by droplets and close contact. Although clinical criteria have been proposed, the diagnosis of streptococcal sore throat relies on a throat culture, direct antigen detection, nucleic acid probe, or nucleic acid amplification tests. About one third of children complaining of sore throat have a throat culture positive for *S. pyogenes*. An estimated 600 million cases of pharyngitis caused by GAS occur each year worldwide.

Case check 15.2

The patient in the Case in Point presented with classic symptoms of streptococcal pharyngitis, although other infectious agents can produce similar symptoms. M protein is the main virulence factor in *S. pyogenes*. Other virulence factors include fibronectin-binding protein (protein F); lipoteichoic acid; hyaluronic acid capsule; and extracellular products, including hemolysins, toxins, and enzymes. Antibodies to oxygen-labile SLO can be used to diagnose recent infection.

Pyoderma infections

Skin or pyoderma infections with GAS result in impetigo, cellulitis, erysipelas, wound infection, or arthritis. **Impetigo**, a localized skin disease, begins as small (nonbullous) vesicles that progress to weeping lesions. The lesions crust over after several days. Impetigo is usually seen in young children (2 to 5 years of age) and affects exposed areas of the skin. Inoculation of the organism occurs through minor abrasions or insect bites. *Staphylococcus aureus* produces a bullous form of impetigo. **Erysipelas** is a rare infection of the skin and subcutaneous tissues observed frequently in older adult patients. It is characterized by an acute spreading skin lesion that is intensely erythematous with a plainly demarcated but irregular edge. **Cellulitis** can develop following deeper invasion by streptococci. The infection can be serious or life-threatening with bacteremia or sepsis. In patients with peripheral vascular disease or diabetes, cellulitis can lead to gangrene.

Infection with strains of *S. pyogenes* that produce streptococcal pyrogenic exotoxins can result in scarlet fever. Strains of *S. pyogenes* infected with the temperate bacteriophage T12 produce streptococcal pyrogenic exotoxins. Scarlet fever, which appears within 1 to 2 days after bacterial infection, is characterized by a diffuse red rash that appears on the upper chest and spreads to the trunk and extremities. The rash disappears over the next 5 to 7 days and is followed by desquamation.

Necrotizing fasciitis

GAS have been associated with necrotizing fasciitis (NF), an invasive infection characterized by rapidly progressing inflammation and necrosis of the skin, subcutaneous fat, and fascia. The most common serotypes associated with NF are M1, M3, M12, and M28. Although uncommon, NF is a life-threatening infection. The Centers for Disease Control and Prevention (CDC) tracks NF caused by GAS. Since 2010, approximately 700 to 1100 cases of NF have occurred annually in the United States; however, this is likely an underestimation as some cases are probably not reported. NF occurs most frequently in individuals who have other health problems.

Death can be prevented if early intervention is instituted; the mortality rate may reach greater than 70% if the infection is left untreated. Many different bacteria can cause destruction of the soft tissue in this manner, a clinical feature that has been described as *flesh-eating disease*. Depending on which organisms are cultured, NF may be categorized as type 1, 2, or 3. A polymicrobial infection from which aerobic and anaerobic bacteria are recovered is categorized as type 1 NF. **Type 2 NF consists of only GAS.** Type 3 is gas gangrene or clostridial myonecrosis. A variant of type 1 NF is saltwater NF, in which an apparently minor skin wound is contaminated with saltwater containing a *Vibrio* species, such as *Vibrio vulnificus*.

Cases of NF were described in the 18th century, but the term was not conceived until 1952. In addition to *flesh-eating bacteria syndrome*, other terms for NF have included *suppurative fasciitis*, *hospital gangrene*, and *necrotizing erysipelas*. NF can occur as a result of trauma, such as burns and lacerations. The break in the skin may become the portal of entry for the bacteria. However, NF infections caused by GAS occur in young, healthy individuals, and a break in the skin that served as portal of entry is not found in many cases. Good wound care is important in minimizing the risk of NF.

Streptococcal toxic shock syndrome

The reemergence of streptococcal toxic shock syndrome (TSS) in the 1980s remains largely unexplained. Streptococcal TSS is a condition in which the entire organ system collapses, leading to death. The exact portal of infection is unknown for most cases of streptococcal TSS, **although minor injuries or surgical procedures have been implicated.** The initial streptococcal infection is often severe (e.g., pharyngitis, peritonitis, cellulitis, wound infections), and the symptoms that develop are similar to symptoms of staphylococcal TSS. Patients are often bacteremic and have NF.

GAS associated with streptococcal TSS produce a streptococcal pyrogenic exotoxin, notably SpeA. These toxins likely play a major role in the pathogenesis of this disease functioning as superantigens, leading to overstimulation of the immune response, resulting in cytokine production (cytokine storm). Other virulence factors, such as SLO and various cell wall antigens, can also contribute to toxic shock. Isolates with M1 and M3, which account for about 50% of cases, are the most common strains associated with streptococcal TSS. Young children, especially children with chickenpox (varicella), and older adults seem to be at greatest risk.

Poststreptococcal sequelae

Two serious complications, or sequelae, of **GAS disease are rheumatic fever and acute glomerulonephritis.** Rheumatic fever typically follows *S. pyogenes* pharyngitis. It is characterized by fever and inflammation of the heart, joints, blood vessels, and subcutaneous tissues. Attacks usually begin within **1 month after infection.** The most serious result is chronic, progressive damage to the heart valves. Repeated infections can produce further valve damage. In some instances, antibodies from streptococcal sequelae cross-react with the brain to produce a neurologic manifestation of **acute rheumatic fever called Sydenham chorea.** Rheumatic fever is rare in most developed countries, and it is no longer a reportable disease in the United States. Acute rheumatic fever and its chronic sequela, rheumatic heart disease, remain problematic in developing countries and in some poor populations in

industrialized countries. The pathogenesis of rheumatic fever is caused by antigenic cross-reactivity between streptococcal antigens and heart tissue.

Acute glomerulonephritis sometimes occurs after a cutaneous or pharyngeal infection. It is more common in children than in adults. The pathogenesis appears to be immunologically mediated. Circulating immune complexes are found in the plasma of patients with acute glomerulonephritis, and it is postulated that these antigen-antibody complexes deposit in the glomeruli. Complement is subsequently fixed, and an inflammatory response causes damage to the glomeruli, resulting in impairment of kidney function.

GAS are susceptible to penicillin, which remains the drug of choice for treatment. For patients who are allergic to penicillin, erythromycin can be used. For patients who have a history of rheumatic fever, prophylactic doses of penicillin are given to prevent recurrent infections that might cause additional damage to the heart valves.

Laboratory diagnosis

An essential step in the diagnosis of streptococcal pharyngitis is proper sample collection. The tongue should be depressed, and the swab rubbed over the posterior pharynx and each tonsillar area. If exudate is present, it should also be touched with the swab. Care should be taken to avoid the tongue and uvula. **Examination of Gram stains of upper respiratory tract specimens or skin swabs is of little value because these areas have considerable amounts of gram-positive cocci as part of the normal bacterial biota.**

Transport media are not required for normal conditions. The organism is resistant to drying and can be recovered from swabs several hours after collection. **Desiccation can enhance the recovery of *S. pyogenes* by causing the loss of viability of normal microbiota.** An SBA plate is inoculated and streaked for isolation. Incubation should be at 35°C either in ambient air or **under anaerobic conditions.** The normal respiratory microbiota tends to overgrow β -hemolytic streptococci when incubated in increased concentration of CO₂. Several **selective media**, such as SBA with phenylethyl alcohol, sulfamethoxazole (SMZ), or colistin and polymyxin B found in some formulations of *Streptococcus* selective agar, have been recommended for better recovery of β -hemolytic streptococci from throat cultures. The plate is observed after 24 hours for the presence of β -hemolytic colonies. If none are found, incubation should continue for an additional 24 hours before the culture is reported as negative. False-negative results can occur from overgrowth of normal microbiota and lack of β -hemolysis.

Colonies of *S. pyogenes* on SBA are small, transparent, and smooth with a well-defined area of β -hemolysis. Gram stain reveals gram-positive cocci with some short chains. Suspect colonies can be Lancefield-typed using serologic methods, which gives a definitive, rapid identification, or biochemical tests can be performed. The correlation between presumptive identification using biochemical methods and the rapid definitive serologic method is high. Most clinical laboratories choose to use serologic methods. Key tests include bacitracin susceptibility or **pyrrolidonyl- α -naphthylamide (PYR) hydrolysis.** ***S. pyogenes* is susceptible to bacitracin and hydrolyzes PYR,** whereas most other β -hemolytic groups are resistant to bacitracin and are PYR-negative. The best way to differentiate group C and G streptococci from GAS

is Lancefield typing. Group C streptococci are generally sensitive to bacitracin. Bacitracin resistance is variable among group G streptococci.

When the origin of an isolate is not the throat (i.e., blood or sputum) and serologic identification is not used, the following tests should be part of the early identification scheme in addition to PYR and bacitracin susceptibility testing: hippurate hydrolysis; Christie, Atkins, Munch-Peterson (CAMP) test; bile esculin test; and growth in 6.5% sodium chloride (NaCl) broth. The reactions of some catalase-negative, gram-positive cocci in various biochemical tests are outlined in Table 15.3. Immunologic tests used to detect infection with *S. pyogenes* include ASO, anti-DNase, antistreptokinase, and antihyaluronidase titers.

Rapid antigen detection tests (RADTs) may be used to diagnose GAS pharyngitis at the point of care. These rapid tests detect *S. pyogenes*-specific cell wall antigens by enzyme immunoassays or optical immunoassays. Sensitivities of 90% in adults and 80% to 90% in children have been reported. In children, a negative RADT result should be confirmed with throat cultures. Rapid polymerase chain reaction (PCR) assays such as the Lyra Direct Strep assay (Quidel Corporation, San Diego, CA), Liat Strep A assay (Roche Diagnostics, Indianapolis, IN), and Simplexa Group A Strep Direct test (Focus Diagnostics, Cypress, CA), among others, are available for the detection of *S. pyogenes* in throat swabs in the hospital setting.

Commercial systems for direct identification of streptococci from blood cultures include the Verigene gram-positive blood-culture (BC-GP) nucleic acid test (Luminex, Austin, TX) and the FilmArray blood culture identification (BCID) panel (bioMérieux, Durham, NC). Misidentification of *S. dysgalactiae* subsp. *equisimilis* as *S. pyogenes* has been reported with matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry bacterial identification systems (Bruker, Billerica, MA).

Streptococcus agalactiae

Antigenic structure

All strains of *S. agalactiae* have the group B-specific antigen, an acid-stable polysaccharide located in the cell wall. *S. agalactiae* is the only species that expresses group B antigen. Additionally, there are nine recognized capsular polysaccharide serotypes. These type-specific antigens can be detected by precipitin tests. Serotypes Ia, Ib, and II contain a terminal residue of sialic acid, which is weakly immunogenic and can inhibit activation of the alternative complement pathway.

Virulence factors

The capsule is an important virulence factor in infections with group B streptococci (GBS). Antibodies against the type-specific antigens protect mice against strains of *S. agalactiae* with the homologous polysaccharide in the capsule. The capsule prevents phagocytosis but is ineffective after opsonization. Sialic acid appears to be the most significant component of the capsule and a critical virulence determinant. Studies with mutant strains of *S. agalactiae* showed that the loss of capsular sialic acid was associated with loss of virulence. Other products produced by *S. agalactiae* include a hemolysin,

CAMP factor, neuraminidase, DNase, hyaluronidase, and protease. No evidence exists that any of these products play a role in the virulence of this organism.

Clinical infections

GBS were known for many years as the cause of mastitis in cattle. It was not until Lancefield defined streptococcal classification in the 1930s that their role in human disease was recognized. *S. agalactiae* was identified in the 1970s as a significant cause of invasive disease in the newborn, and it remains so today. GBS are the leading cause of death in infants in the United States, although the incidence decreased dramatically from the 1990s to 2008. Two clinical syndromes are associated with neonatal GBS disease: early-onset infection (<1 week of age) and late-onset infection (at least 1 week of age to about 3 months of age). Early-onset disease accounts for about 80% of the clinical cases in newborns and is caused by vertical transmission of the organism from the mother. Colonization of the vagina and rectal area with GBS is found in 10% to 30% of pregnant women.

Most infections of infants occur in the first 3 days after birth, usually within 24 hours. This infection is commonly associated with obstetric complications, prolonged rupture of membranes, and premature birth. Early-onset infection often manifests itself as pneumonia and sepsis, and late-onset infection manifests itself as meningitis and sepsis. Serotype III GBS clone ST-17 is reported to be responsible for the majority of the early- and late-onset infections worldwide. The mortality rate in GBS-infected infants is high, and death usually occurs if treatment is not started quickly.

The most important determining factor in early-onset infection is the presence of GBS in the vagina of the mother. It is recommended that all pregnant women be screened for GBS at 35 to 37 weeks' gestation. Screening methods are discussed later. As mentioned earlier, late-onset infection occurs between 1 week and 3 months after birth and usually manifests itself as meningitis. This infection is uncommonly associated with obstetric complications. Also, the organism is rarely found in the mother's vagina before birth. The mortality rate is considerably less than the mortality rate associated with early-onset disease, but it is high enough to be of serious concern.

The incidence of GBS infections decreases dramatically after the neonatal period. In adults, the infection affects two patient types. One type is a young, previously healthy woman who becomes ill after childbirth or abortion; endometritis and wound infections are most common. In addition, tricuspid valve endocarditis is sometimes observed in young women undergoing obstetric procedures. The second type of patient is an older adult with a serious underlying disease or immunodeficiency. In this patient population, GBS infection manifests itself as skin and soft tissue infections, intraabdominal abscess, bacteremia, or pneumonia. Other infections such as endocarditis and urinary tract infections (UTIs) have also been reported.

The drug of choice for treating GBS infections is penicillin, although GBS are less susceptible to penicillin than GAS. The clinical response to antimicrobial therapy is often poor despite the heavy doses given. Some clinicians recommend a combination of ampicillin and an aminoglycoside for treating GBS infections. Despite the increased use of prophylactic

Table 15.3 Biochemical Identification of *Streptococcus* and Similar Organisms

Characteristic	<i>S. pyogenes</i>	<i>S. agalactiae</i>	Other β-Hemolytic Species ^a	<i>Enterococcus</i>	Group D Streptococci	<i>S. pneumoniae</i>	Viridans Streptococci	<i>Aerococcus</i>	<i>Pediococcus</i>	<i>Leuconostoc</i>
Hemolysis type	β	β	β	α, β, none	α, none	α	α, none	α, none	α, none	α, none
Susceptibility to										
Vancomycin	S	S	S	S(R)	S	S	S	S	R	R
Bacitracin	S	R ^b	R ^b	R	R	S	R ^b	S		
SMZ	R	R	S	R	V	S	S			
Optochin	R	R	R	R	R	S	R			
Hydrolysis of										
Hippurate	–	+	–	– ^b	–	–	– ^b	+	+	
PYR	+	–	–	+	–	–	–	+	–	–
CAMP	–	+	–	–	–	–	–	–	–	–
Leucine aminopeptidase	+	+	+	+	+	+	+	–	+	–
Bile esculin	–	–	–	+	+	–	– ^b	V	+	V
Growth in 6.5% NaCl	–	–	–	+	–	–	–	+	V	V
^a β-Hemolytic groups other than A, B, and D. ^b Exceptions may occur. +, Present; –, absent; <i>CAMP</i> , Christie, Atkins, Munch-Petersen test; <i>PYR</i> , pyrrolidonyl-α-naphthylamide; <i>R</i> , resistant; <i>S</i> , susceptible; <i>SMZ</i> , sulfamethoxazole; <i>S(R)</i> , greater percentage susceptible; <i>V</i> , variable.										

antimicrobials in pregnant women, *S. agalactiae* has remained susceptible to penicillin and cephalosporins; however, increasing minimal inhibitory concentrations (MICs) have been reported.

Laboratory diagnosis

GBS grow on SBA as grayish white mucoid colonies surrounded by a small zone of β -hemolysis (Fig. 15.5). These organisms are gram-positive cocci that form short chains in clinical specimens and longer chains in culture. Presumptive identification is based on biochemical reactions. The most useful tests are hippurate hydrolysis and CAMP tests. These tests enable the organism to be readily differentiated from other β -hemolytic streptococcal isolates. Fig. 15.6 demonstrates how susceptibility to the Bacitracin disk and the CAMP test can be used to differentiate *Streptococcus* spp. The definitive identification can be made by extracting the group antigen and demonstrating agglutination with specific anti-group B antisera.

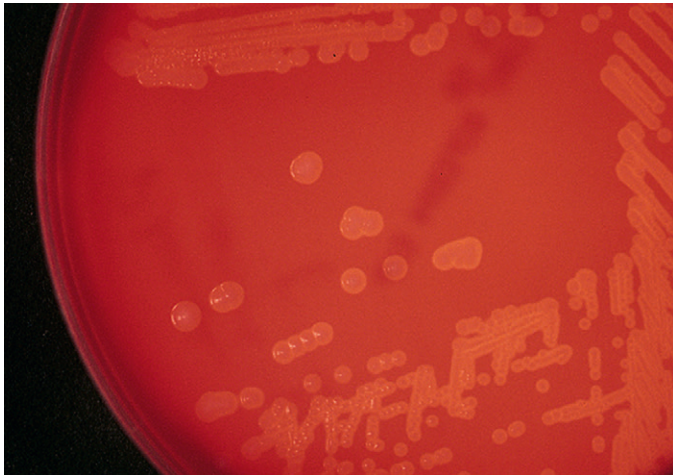


Fig. 15.5 *Streptococcus agalactiae* colony growing on sheep blood agar.

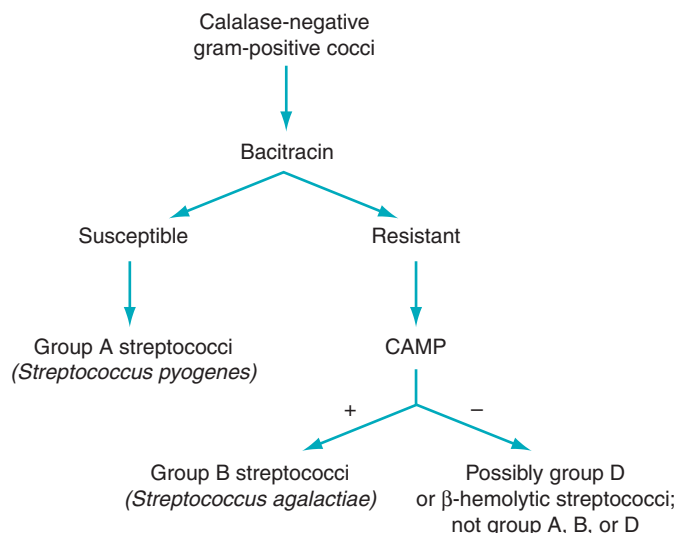


Fig. 15.6 Schematic diagram for differentiation of group A streptococci from group B streptococci. CAMP, Christie, Atkins, Munch-Petersen test.

Detection of GBS in pregnant women is accomplished by collecting vaginal and rectal material with swabs between 35 and 37 weeks' gestation. Samples should be inoculated into selective broth, such as Todd-Hewitt broth containing 10 μ g/mL colistin and 15 μ g/mL nalidixic acid (Lim broth; BD Diagnostic Systems, Sparks, MD). Broth containing 8 μ g/mL gentamicin and 15 μ g/mL nalidixic acid (TransVag broth, Thermo Fisher Scientific, Waltham, MA) may also be used. The inoculated media are incubated at 35°C for 18 to 24 hours before being subcultured to SBA. Numerous nucleic acid amplification tests for nonenriched clinical specimens and culture-enriched specimens are available. The BD MAX GBS Assay (BD Diagnostic Systems) is a fluorescence-based PCR assay that can be used to detect GBS in broth and determine GBS colonization in antepartum women.

Chromogenic agar that changes color if β -hemolytic GBS are present can be used in place of SBA when performing subcultures from broth or used for direct plating of clinical specimens. Cultures are examined for colonies resembling GBS after 24 hours of incubation at 35°C in an incubator containing 5% CO₂. Cultures that do not show colonies resembling GBS are incubated for an additional 24 hours.

StrepB Carrot Broth (SCB; Hardy Diagnostics, Santa Maria, CA), which can be substituted for Lim or TransVag broth, was shown to have improved diagnostic value and efficiency. Vaginal or rectal swabs are placed into SCB and incubated for 18 to 24 hours at 35°C. β -Hemolytic GBS often produce an orange or red pigment in SCB after incubation for 6 hours. Further identification is unnecessary. Isolates that are non-hemolytic do not produce a color change, requiring that the broth be subcultured. Approximately 4% of invasive isolates are nonhemolytic.

The CDC recommends universal vaginal and rectal screening of all pregnant women during the late third trimester. Women who test positive should receive prophylactic antimicrobial therapy. Additional women who should be treated include those who had a urine culture positive for GBS any time during the pregnancy, a history of an infant with GBS disease, and an unknown GBS status at the time of delivery (culture was not done). The CDC also recommends screening pregnant women for asymptomatic bacteriuria. GBS should be identified and reported to the primary care provider when the colony count is greater than or equal to 10⁴/mL in either pure culture or mixed with a second microorganism. These preventive measures help reduce the risk of early-onset GBS infection but are not likely to affect late-onset infections significantly.

Group C and G streptococci

In the most recent classification of β -hemolytic streptococci, isolates from humans that belong to Lancefield groups C and G are subdivided into large-colony (diameter >0.5 mm after 24 hour incubation) and small-colony forms (diameter <0.5 mm after 24 hour incubation). The large-colony-forming isolates with group C and G and sometimes group A and L antigens are classified with the pyogenic streptococci. The large-colony-forming β -hemolytic isolates with group A, C, G, or L antigens belong to *S. dysgalactiae* subsp. *equisimilis*. The small-colony-forming β -hemolytic isolates with group A, C, F, or G antigens belong to the *S. anginosus* group, which are included in the viridans streptococci, discussed later.

Clinical infections by *S. dysgalactiae* subsp. *equisimilis*, although uncommon, have involved several body sites and are thought to be uncommon in domestic animals. The spectrum of infections resembles that of *S. pyogenes* and includes upper respiratory tract infections, skin infections, soft tissue infections, and invasive infections such as NF. Because several antigenic groups are included within the species, serotyping of *S. dysgalactiae* to determine the species is complex. *S. equi* subsp. *zooepidemicus*, which also expresses C antigen, is primarily an animal pathogen rarely isolated from humans. It has been associated with cases of glomerulonephritis and rheumatic fever after infections.

Streptococcus pneumoniae

Antigenic structure

Also known as *pneumococcus*, *S. pneumoniae* is isolated from a variety of clinical specimens. *S. pneumoniae* is a member of the *S. mitis* group, but it is much more virulent than other members of the group. The cell wall of *S. pneumoniae* contains an antigen, referred to as C substance, that is similar to the C carbohydrate of the various Lancefield groups. A β -globulin in human serum, called C-reactive protein (CRP), reacts with C substance to form a precipitate. This is a chemical reaction and not an antigen-antibody combination. The amount of CRP increases during inflammation and infection.

S. pneumoniae can express 1 of over 100 different capsular types based on chemical variations of the capsular polysaccharide. Antibody directed against the capsular antigen is protective. Isolates from certain sources—for example, cases of lobar pneumonia—show a predominance of particular capsular types. The capsule is antigenic and can be identified with appropriate antisera in the Neufeld test. In the presence of specific anticapsular serum, the capsule swells (Quellung reaction). This reaction not only allows identification of *S. pneumoniae* but also serves to specifically serotype the isolate.

Virulence factors

The characteristic of *S. pneumoniae* that is clearly associated with virulence is the capsular polysaccharide. Strains that have lost the ability to produce a capsule are nonpathogenic. In addition, opsonization of the capsule renders the organism avirulent. Several toxins are produced, including a hemolysin, an immunoglobulin A protease, neuraminidase, and hyaluronidase. None of these have been shown to have a role in disease production.

Clinical infections

S. pneumoniae is a common isolate in the clinical microbiology laboratory. It is an important human pathogen that causes pneumonia, sinusitis, otitis media, bacteremia, and meningitis. *S. pneumoniae* is the most frequently encountered isolate in children younger than 3 years of age with recurrent otitis media. *S. pneumoniae*, the leading cause of bacterial pneumonia, is especially prevalent in older adults and in patients with underlying disease. Of the over 100 capsular serotypes, about one fourth account for most pneumococcal pneumonia cases. In a U.S. study from 2015 to 2016, 26 serotypes accounted for 98% of the *S. pneumoniae* isolates. Despite being a component of pneumococcal vaccines, serotype 3 was the most common

isolate (657/5334; 12.3%). Various studies have indicated that serotype 3 is linked to some of the most severe infections, such as empyema, bacteremia, cardiotoxicity, and meningitis, with a fatality rate of 30% to 47%.

For an individual to contract pneumococcal pneumonia, the organism must be present in the nasopharynx, and the individual must be deficient in the specific circulating antibody against the capsular type of the colonizing strain of *S. pneumoniae*. After initial colonization, the bacteria can persist for weeks or months without causing disease. In some individuals, invasive disease occurs that leads to community-acquired pneumonia.

Pneumonia resulting from *S. pneumoniae* is not usually a primary infection but is rather a result of disturbance of the normal defense barriers. Predisposing conditions, such as alcoholism, anesthesia, malnutrition, and viral infections of the upper respiratory tract, can lead to pneumococcal disease in the form of lobar pneumonia. Older adults are also at increased risk for infection. The infection begins with aspiration of respiratory secretions, which often contain pneumococci. In the alveoli, the organisms stimulate an outpouring of fluid, which facilitates the spread of the organism to adjacent alveoli. The process stops when the fluid reaches fibrous septa that separate the major lung lobes. This accounts for the “lobar” distribution of the infection—hence the name. Pneumococcal pneumonia is characterized by sudden onset of chills, dyspnea, and cough.

Pneumonia may be complicated by a pleural effusion that is usually sterile (empyema). The laboratory might receive fluid from a pleural aspirate for culture. An infected effusion contains many white blood cells and pneumococci, which are visible on Gram stain. Even with antimicrobial therapy, mortality is relatively high (5% to 10%); however, without therapy, the mortality rate approaches 50%. Pneumococcus causes bacterial meningitis in all age groups. Meningitis usually follows other *S. pneumoniae* infections, such as otitis media or pneumonia. The course of the disease is rapid, and the mortality rate is near 40%. Direct smears of the cerebrospinal fluid often reveal leukocytes and numerous gram-positive cocci in pairs. Pneumococci may also be involved in other infections, such as endocarditis, peritonitis, and bacteremia. Bacteremia often occurs during the course of a serious infection. Consequently, samples for blood culture are often taken simultaneously with sputum or a fluid aspirate.

Currently, three pneumococcal conjugate vaccines (PCVs) are available. PCV13, protecting against 13 serotypes commonly affecting children, is composed of purified polysaccharides conjugated to a diphtheria protein and is approved for use in children. This vaccine is part of the routine pediatric immunization schedule encompassing four doses beginning at 2 months of age. PCV15 is recommended for individuals 19 to 64 years of age, and PCV20 is recommended for people 65 years or older who have not previously received a pneumococcal conjugate vaccine. The 23-valent pneumococcal polysaccharide vaccine (PPSV23), composed of 23 purified capsular polysaccharides, is used for adults. Vaccination is recommended for all individuals older than 65 years of age or individuals with a long-term health problem (e.g., asplenic individuals or patients with diabetes, leukemia, lymphoma, or human immunodeficiency virus infection). Typically, only a single dose is needed. The vaccine has been successful in reducing the incidence and severity of pneumococcal disease. Epidemiologic surveys report an increase in infections resulting from serotypes not covered by the vaccine serotypes.

Laboratory diagnosis

The cells characteristically seen on Gram stain appear as gram-positive cocci in pairs (diplococci). The ends of the cells are slightly pointed, giving them an oval or lancet shape (Fig. 15.7). The cocci can occur singly or in short chains but most often are seen in pairs. As the culture ages, the Gram stain reaction becomes variable, and gram-negative cells are seen. The capsule can be demonstrated by using a capsule stain.

The nutritional requirements of *S. pneumoniae* are complex. Media such as brain-heart infusion agar, trypticase soy agar with 5% sheep RBCs, or chocolate agar are necessary for good growth. Some isolates require increased CO₂ concentration for growth during primary isolation. Isolates produce a large zone of α -hemolysis on SBA surrounding the colonies. Young cultures have a round, glistening, wet, mucoid, dome-shaped appearance (Fig. 15.8). As the colonies become older, autolytic changes result in a collapse of each colony's center, giving it the appearance of a coin with a raised rim. The tendency of *S. pneumoniae* to undergo autolysis can make it difficult to keep isolates alive. Clinical isolates and stock cultures require frequent subculturing (every 1 to 2 days) to ensure viability.

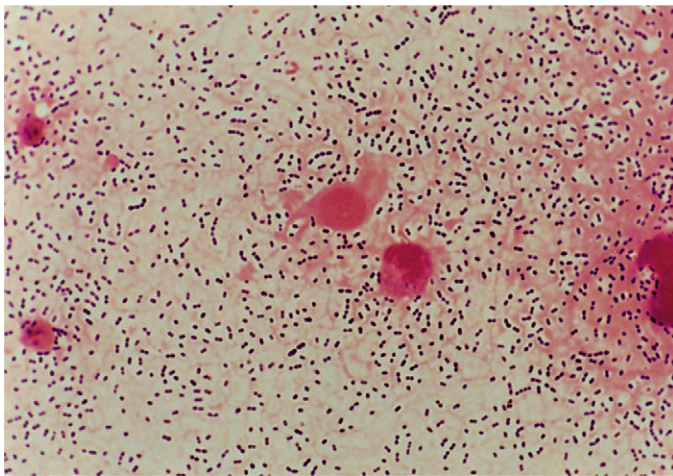


Fig. 15.7 Gram stain of *Streptococcus pneumoniae*. Direct smear of sputum from a patient with pneumonia caused by *S. pneumoniae*. The clear, nonstained area around the organism represents the capsule. ($\times 1000$).

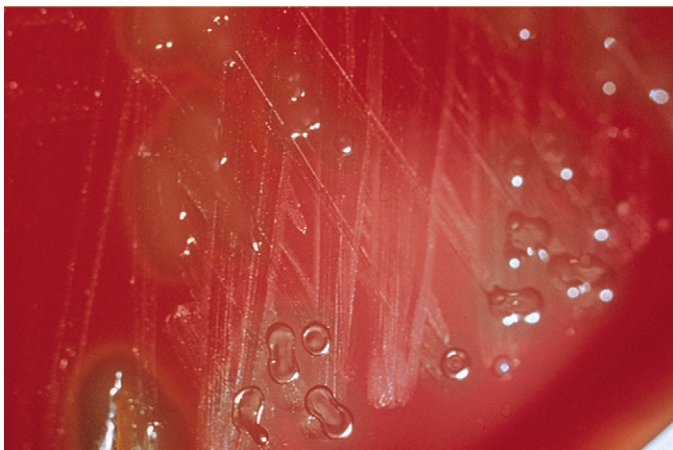


Fig. 15.8 *Streptococcus pneumoniae* colonies on sheep blood agar. The colonies demonstrate a characteristic mucoid appearance and a concave center.

The colonies may closely resemble colonies of the viridans streptococci, and the greatest concern in the laboratory diagnosis is distinguishing *S. pneumoniae* from the viridans streptococci. However, a presumptive differentiation is not difficult to make. Optochin susceptibility and bile solubility tests are used to accomplish this; the optochin susceptibility test is more commonly used. The optochin susceptibility test takes advantage of the fact that *S. pneumoniae* is susceptible to optochin, whereas other α -hemolytic species are resistant. The bile solubility test determines the lysis of *S. pneumoniae* in the presence of bile salts. It correlates with optochin susceptibility; that is, *S. pneumoniae* isolates are optochin susceptible and bile soluble. The Phadebact pneumococcus test (MKL Diagnostics, Sollentuna, Sweden), is a rapid test for the identification of culture isolates. The coagglutination assay uses anticapsular antibodies produced in rabbits. Currently, many clinical microbiology laboratories are starting to use MALDI-TOF mass spectrometry for routine bacterial identification. Issues with differentiation of *S. pneumoniae* from closely related viridans species such as *S. mitis* have been observed with MALDI-TOF mass spectrometry.

Results from the *S. pneumoniae* antigen detection test in urine, in conjunction with clinical and radiologic findings, can aid diagnosis. The BinaxNOW *S. pneumoniae* antigen card (Alere, Waltham, MA) is an immunochromatographic test based on the detection of a C polysaccharide common to all *S. pneumoniae*. The test has a specificity greater than 90% and sensitivity between 50% and 80% in urine. Because of low sensitivity, less than 30%, direct antigen detection tests are not recommended on cerebrospinal fluid. The FilmArray BCID panel is a qualitative multiplexed nucleic acid-based diagnostic test that can be used for rapid identification of *S. pneumoniae* in blood culture.

Antimicrobial resistance

The susceptibility of *S. pneumoniae* to penicillin and macrolides has varied with time, geographic region, and country. Because most isolates are susceptible to penicillin when administered parenterally, penicillin remains the drug of choice for treating pneumococcal infections. Penicillin resistance is caused by altered penicillin-binding proteins. Penicillin-resistant pneumococci are reported to show resistance to other classes of drugs, such as β -lactams, macrolides, and tetracyclines. Resistance to extended-spectrum cephalosporins, such as ceftriaxone and cefotaxime, has also increased, although these agents have been used successfully in the treatment of serious infections caused by penicillin-resistant pneumococci.

Multidrug-resistant *S. pneumoniae*, defined as pneumococcal isolates resistant in vitro to two or more classes of antimicrobial agents, has been reported and can occur in the presence or absence of penicillin resistance. Pneumococcal strains that exhibit resistance to various antimicrobial agents, such as erythromycin, tetracycline, chloramphenicol, and trimethoprim-sulfamethoxazole, have emerged.

Viridans streptococci

Viridans streptococci are constituents of the normal microbiota of the upper respiratory tract, the female genital tract, and the gastrointestinal tract. The term *viridans* means "green," referring to the α -hemolysis many species exhibit. However,

β -hemolytic and nonhemolytic species are also classified as viridans streptococci. **Viridans streptococci are fastidious, with some strains requiring CO₂ for growth.**

More than 30 species are now recognized. The current classification assigns streptococcal species in the viridans group to one of five groups: (1) *S. mitis* group (including *S. mitis*, *S. pneumoniae*, *S. pseudopneumoniae*, *S. sanguinis*, and *S. oralis*); (2) *S. mutans* group (including *S. mutans* and *S. sobrinus*); (3) *S. salivarius* group (including *S. salivarius* and *S. vestibularis*); (4) *S. bovis* group (including *S. equinus*, *S. gallolyticus*, and *S. infantarius*); and (5) *S. anginosus* group (including *S. anginosus*, *S. constellatus*, and *S. intermedius*). Several species remain unclassified. Organisms of the *S. anginosus* group can possess Lancefield group A, C, F, G, or N antigen and in some instances may not be groupable. The organisms also can cross-react with other grouping antisera. Thus identification using Lancefield antisera is of little value. However, a small-colony-forming streptococcus positive for group F antigen isolated from a human specimen is likely a member of the *S. anginosus* group. The *S. salivarius* group and *S. bovis* group have some similar characteristics.

The *S. bovis* group belong to group D streptococci. The *S. bovis* group and the enterococci possess the group D antigen. *S. bovis* is no longer a valid species name. DNA studies found that *S. bovis* and *S. equinus* were the same species, and the earlier species name, *S. equinus*, was adopted. However, current methodologies have identified all human isolates of the *S. bovis* group to be *S. gallolyticus*. Until the mid-1980s, group D streptococci were subdivided into the enterococcal and nonenterococcal groups, with the understanding that organisms found in the intestinal tract were part of the enterococcal group. Both groups were bile esculin positive, but the non-enterococcal organisms would not grow in a nutrient broth with 6.5% NaCl. As more became known about the molecular characteristics of each of these subgroups, the enterococcal group was placed into a new genus, *Enterococcus*, but the non-enterococcal group remained part of group D streptococci.

Clinical infections

Viridans streptococci are oropharyngeal commensals that are regarded as opportunistic pathogens. Although their virulence is low, they cause disease if host defenses are compromised. Viridans streptococci are the most common cause of subacute bacterial endocarditis, a condition associated with a transient bacteremia. Viridans streptococcal bacteremia is more common in children than in adults and is usually more prevalent in patients with hematologic malignancies. Fatal cases have been characterized by fulminant cardiovascular collapse or meningitis. Generally, the course of endocarditis is very slow; symptoms may be present for weeks or months. Individuals whose heart valves have been damaged by rheumatic fever are especially susceptible to endocarditis from viridans streptococci.

Besides bloodstream infections, viridans streptococci play a role in oral infections such as gingivitis and dental caries (cavities). They have also been implicated in meningitis, abscesses, osteomyelitis, and empyema. Although viridans streptococci are frequently isolated in association with other bacteria from bronchial brushing, their role in bacterial pneumonia is unclear. Most viridans streptococcal infections are treated with penicillin, although some resistant strains have been reported.

Most members of the *S. anginosus* group are considered part of the normal oral and gastrointestinal microbiota; however, they have been associated with abscess formation in the oropharynx, brain, and peritoneal cavity. Members of the *S. anginosus* group have been isolated from bacteremic patients with invasive pyogenic infections with the tendency to form abscesses. *S. constellatus* subsp. *pharyngis* has been linked to pharyngitis. All clinically significant species of the *S. sanguinis* group have been isolated from patients with endocarditis.

The *S. mitis* group is normally found in the oral cavity, gastrointestinal tract, and female genital tract. Members also can be found as transient normal microbiota of the skin. Although they can be isolated from the blood of asymptomatic individuals, they are the most common isolates associated with bacterial endocarditis in native valves and, less frequently, in prosthetic valve infections. *S. salivarius* and *S. vestibularis* have been isolated from human specimens. *S. salivarius* has been linked to bacteremia, endocarditis, and meningitis, whereas *S. vestibularis* has not been associated with disease. *S. pseudopneumoniae* isolates have been observed in patients with respiratory tract infection and chronic obstructive pulmonary disease (COPD). Unlike *S. pneumoniae*, *S. pseudopneumoniae* do not have a capsule and are insoluble in bile. However, they demonstrate resistance or indeterminate susceptibility to optochin when incubated in 5% CO₂ and susceptibility to optochin when incubated in ambient air.

Among the viridans streptococci, the *S. mutans* group is the most commonly isolated, and members are usually recovered from the oral cavity. *S. mutans* is the primary contributor to dental caries. It is also the most common member of the *S. mutans* group associated with bacteremia. Members of the *S. bovis* group are often encountered in blood cultures of patients with bacteremia, septicemia, and endocarditis. The presence of *S. gallolyticus* subsp. *gallolyticus* in blood cultures has a high correlation with gastrointestinal carcinoma.

Virulence factors

Virulence factors that characterize the pathogenicity of viridans streptococci have not been well established. A polysaccharide capsule and cytolysin have been identified in some members of the *S. anginosus* group. Besides these, extracellular dextran and cell surface-associated proteins (adhesins) have been implicated in adherence and colonization of these organisms in endocarditis. Group C and G streptococci possess M proteins with similarity to those of GAS. Some of them also produce extracellular enzymes such as SLO, hyaluronidase, and DNase.

Laboratory diagnosis

It is extremely difficult to identify isolates of the viridans group to the species level; clinical laboratories should be satisfied to place isolates into one of the five groups. All members are PYR negative and **leucine aminopeptidase (LAP)** positive. Viridans streptococci show typical *Streptococcus* characteristics on Gram stain. On SBA, colonies are small and are surrounded by a zone of α -hemolysis; some isolates are β -hemolytic or nonhemolytic. The lack of β -hemolysis separates the viridans streptococci from groups A, B, C, and G. The differentiation of α -hemolytic viridans streptococci from *S. pneumoniae*, also α -hemolytic, is based on optochin sensitivity, bile solubility, or anticapsular antibody tests.

Table 15.4 Characteristics of Viridans Streptococci^a

	Mannitol	Sorbitol	Voges-Proskauer	Arginine Hydrolysis	Esculin Hydrolysis	Urease	Hemolytic Pattern
<i>S. anginosus</i> group	—	—	+	+	+	—	α, β, Non
<i>S. bovis</i> group	v	—	+	—	v	—	Non
<i>S. mitis</i> group	—	v	—	v	v	—	α
<i>S. mutans</i> group	+	+	+	—	+	—	α, β, Non
<i>S. salivarius</i> group	—	—	+	—	v	v	α

^aAll viridans streptococci are leucine aminopeptidase positive and pyrrolidonyl-α-naphthylamide negative.

+, Positive test result; —, negative test result; v, variable test result; Non, nonhemolytic.

From Spellerberg, B., et al. (2019). *Streptococcus*. In K. C. Carroll, et al. (Eds.), *Manual of clinical microbiology* (12th ed., p. 399). Washington, DC: ASM Press.

The ability to ferment sugars, the Voges-Proskauer (VP) reaction, β-D-glucuronidase activity, and hippurate hydrolysis are used for the differentiation of species within the viridans group. Some commercial multitest kits can identify more commonly isolated species; however, not all species are part of the manufacturers' databases. The hemolytic patterns and some biochemical tests that distinguish members of the viridans group are shown in Table 15.4.

S. anginosus is composed of strains that may have A, C, F, G, or no Lancefield antigen. These are minute colony types showing α-hemolysis, β-hemolysis, or no hemolysis. When they are β-hemolytic, the zone size is several times the size of the colony. When they are growing in pure culture or in high concentration, a characteristic sweet odor of honeysuckle or butterscotch may be present.

Two groups that might be confused with each other are the genus *Enterococcus* and group D streptococci. Members of the *S. bovis* group express the D antigen. Both group D streptococci and enterococci are usually nonhemolytic (Fig. 15.9), although occasionally enterococcal isolates can be α-hemolytic or β-hemolytic. The differentiation of nonhemolytic streptococci is outlined in Fig. 15.10. Both enterococci and group D streptococci are bile esculin positive. Growth in 6.5% NaCl broth differentiates *Enterococcus* from viridans streptococci that fail to grow (Fig. 15.11). In addition, group D streptococci can be separated from *Enterococcus* with the PYR test; group D streptococci test negative, whereas enterococci test positive. It is important to distinguish group D streptococci from *Enterococcus* because group D streptococci are generally susceptible to penicillin, whereas *Enterococcus* organisms are usually resistant. Some strains of *S. salivarius* can be misidentified as members of the *S. bovis* group because a significant number of *S. salivarius* isolates are bile esculin positive.

Development of molecular methods such as DNA hybridization and PCR for identification of viridans streptococci has been challenging. Multilocus sequence analysis may be used to identify unknown streptococcal species. MALDI-TOF mass spectrometry microbial systems are being evaluated in clinical laboratories for identification of viridans streptococci and for rapidly distinguishing them from enterococci and *S. pneumoniae*.

Enterococcus

The enterococci consist of gram-positive cocci that are natural inhabitants of the intestinal tracts of humans and animals.

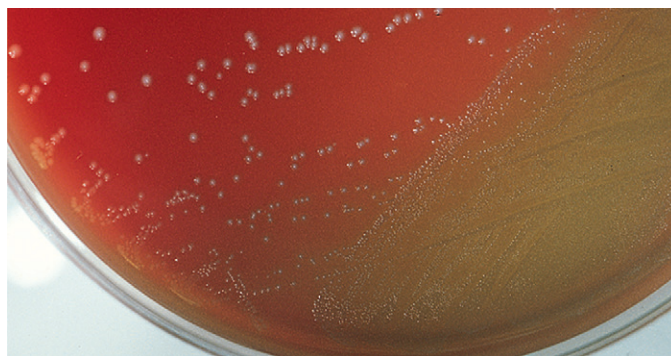


Fig. 15.9 *Enterococcus* species growing on sheep blood agar.

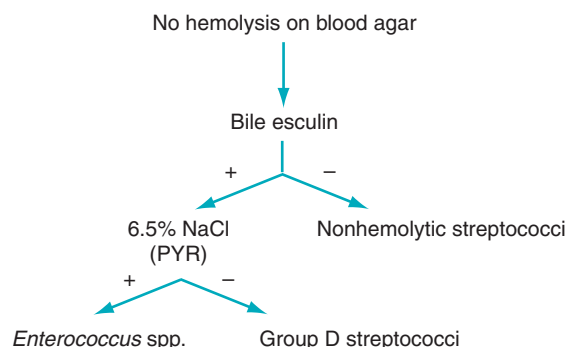


Fig. 15.10 Schematic diagram for identification of nonhemolytic streptococci. PYR, Pyrrolidonyl α-naphthylamide.

The commonly identified species in clinical specimens are *E. faecalis* and *E. faecium*. Other species such as *E. durans*, *E. avium*, *E. casseliflavus*, *E. gallinarum*, *E. hirae*, and *E. raffinosus* are observed occasionally. All species produce the cell wall-associated group D antigen in the Lancefield classification system.

Most enterococci are nonhemolytic or α-hemolytic, although some strains show β-hemolysis. Enterococci sometimes exhibit a pseudocatalase reaction—weak bubbling in the catalase test. Identification to the species level is based on biochemical characteristics. In contrast with streptococci, enterococci have the ability to grow under extreme conditions—for example, in the presence of bile or 6.5% NaCl or at 45°C or alkaline pH. The ability of enterococci to hydrolyze PYR is useful for differentiating them from group D streptococci (Figs. 15.10 and 15.11).

Virulence factors

The virulence factors that contribute to the pathogenicity of enterococci are incompletely understood. The enterococci have a survival advantage over other organisms in that they can grow in extreme conditions and are resistant to multiple antimicrobial agents. Extracellular surface adhesin proteins, extracellular serine protease, and gelatinase of *E. faecalis* are thought to play a role in the colonization of the species and adherence to heart valves and renal epithelial cells. *E. faecalis* also produces a two-subunit toxin, termed *cytolysin*. This toxin shows similarity to bacteriocins produced by other gram-positive bacteria and is expressed by a quorum-sensing mechanism.

Clinical infections

Enterococci are frequent causes of healthcare-associated infections and have been associated with increased use of third-generation cephalosporins to which they are intrinsically resistant. Healthcare-associated UTIs are the most

common, followed by bacteremia. UTI is often associated with urinary catheterization or other urologic manipulations. Prolonged hospitalization is a risk factor for acquiring enterococcal bacteremia. Bacteremia is often observed in patients receiving hemodialysis, immunocompromised patients with a serious underlying disease, or patients who have undergone a prior surgical procedure. Endocarditis from enterococci is seen mainly in older adult patients with prosthetic valves or valvular heart disease. Enterococci account for about 5% to 10% of infections in patients with bacterial endocarditis.

Although they are frequently isolated from intraabdominal or pelvic wound infections, their role in these infections remains contentious. In burn patients, enterococcal wound infection and sepsis resulting from contaminated xenografts have been reported. Rare cases of enterococcal infection of the central nervous system in patients who have had neurosurgery or head trauma and in immunosuppressed patients with enterococcal bacteremia have been reported. Respiratory tract infections from enterococci are also rare and have been reported in severely ill patients who have had prolonged antimicrobial therapy.

Laboratory diagnosis

Standard procedures for collection and transport of blood, urine, or wound specimens should be followed. The specimens should be cultured as soon as possible with minimum delay. Trypticase soy or brain-heart infusion agar supplemented with 5% sheep blood is routinely used to culture enterococci. Enterococci grow well at 35° C in the presence of CO₂ but do not require a high level of CO₂ for growth. If the clinical specimen is obtained from a contaminated site or is likely to contain gram-negative organisms, selective media containing bile esculin azide, colistin–nalidixic acid, phenylethyl alcohol, cephalexin–aztreonam–arabinose, or chromogenic substrates should be used for isolation of enterococci.

Enterococcus spp. are identified based on several biochemical characteristics. *E. faecalis* is easily identified by its ability to grow in the presence of tellurite, as shown in Table 15.5. Commercially available multitest kits may be useful for identification of *E. faecalis*, but they are inadequate for other enterococci. Molecular typing methods, such as pulsed-field gel electrophoresis, contour-clamped homogeneous electric-field electrophoresis, ribotyping, PCR-based typing methods, and whole-genome sequencing, have been used mainly to type

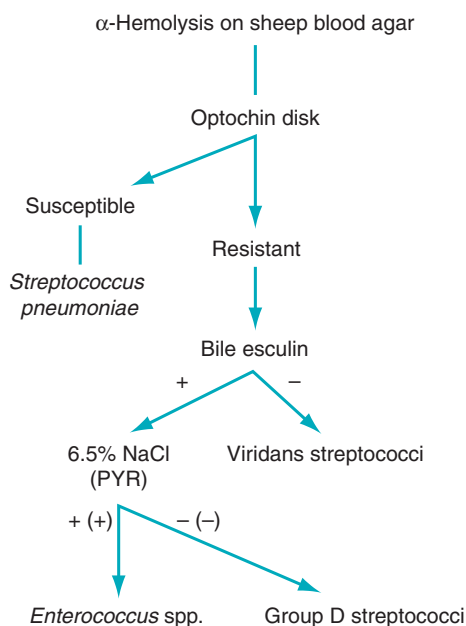


Fig. 15.11 Schematic diagram for differentiation of α-hemolytic streptococci from *Enterococcus*. PYR, Pyrrolidonyl α-naphthylamide.

Table 15.5 Phenotype and Biochemical Characteristics of *Enterococcus* Species

<i>Enterococcus</i> Species	MOT	MAN	SOR	ARA	RAF	TEL	ARG	PYU	MGP
<i>E. faecalis</i>	–	+ ^a	–	–	–	+	+ ^a	+	–
<i>E. faecium</i>	–	+ ^a	–	+	V	–	+	–	–
<i>E. durans</i>	–	–	–	–	–	–	+	–	–
<i>E. avium</i>	–	+	+	+	–	–	–	+	V
<i>E. casseliflavus</i>	+ ^a	+	–	+	+	– ^a	+ ^a	V	+
<i>E. gallinarum</i>	+ ^a	+ ^a	–	+	+	–	+ ^a	–	+
<i>E. raffinosus</i>	–	+	+	+	+	–	–	+	V

^aOccasional exceptions occur.

+, Positive test; –, negative test; v, variable test result; ARA, arabinose; ARG, arginine; MAN, mannitol; MGP, methyl α-D-glucopyranoside; MOT, motility; PYU, pyruvate; RAF, raffinose; SOR, sorbose; TEL, tellurite; V, variable test.

From Teixeira, L. M., et al. (2019). *Enterococcus*. In K. C. Carroll, et al. (Eds.), *Manual of clinical microbiology* (12th ed., p. 418). Washington, DC: ASM Press.

Enterococcus spp. in epidemiologic studies and investigations of vancomycin-resistant enterococci (VRE).

Antimicrobial resistance

Enterococcus spp. show resistance to several commonly used antimicrobial agents; therefore differentiation from *Streptococcus* and susceptibility testing are important. Enterococci have intrinsic or acquired resistance to several antimicrobial agents, including aminoglycosides, β -lactams, and glycopeptides. Because of their low expression of penicillin-binding proteins, *Enterococcus* spp. are resistant to β -lactams. Resistance of enterococci to glycopeptides such as vancomycin and teicoplanin was first described in the late 1980s. Nine vancomycin-resistant phenotypes have been described in enterococci: VanA, VanB, VanC, VanD, VanE, VanG, VanL, VanM, and VanN. The phenotypes VanA, VanB, VanD, and VanM replace the D-Ala-D-Ala termini of peptidoglycan pentapeptide precursors with D-Ala-D-lactate. The phenotypes VanC, VanE, VanG, VanL, and VanN replace the peptidoglycan pentapeptide precursors termini with D-Ala-D-Ser. The VanC phenotype was first observed in *E. gallinarum* and *E. casseliflavus*, which are intrinsically resistant to vancomycin. Of the vancomycin-resistant phenotypes, VanA and VanB phenotypes are most frequently encountered. The VanA phenotype is inducible, carried on a transposon (Tn1546) in the chromosome or plasmid, and characterized by high-level resistance to vancomycin (MIC $>32\mu\text{g/mL}$) and teicoplanin (MIC $>16\mu\text{g/mL}$), whereas the VanB phenotype is also inducible and carried on a transposon Tn5382 (previously Tn1549) in the chromosome or plasmid and characterized by variable levels of resistance to vancomycin and susceptibility to teicoplanin.

VRE is a global problem. *E. faecium* is responsible for the majority of the VRE infections. In the United States, approximately 14% of *E. faecalis* and 87% of *E. faecium* isolated from blood are vancomycin resistant. The frequency of VRE isolates in regions outside the United States range from 14% to 48%. Vancomycin-containing agar and chromogenic VRE media have been used for screening of VRE colonization and for infection control in hospitals. In addition, PCR-based assays have been used to detect VRE.

Streptococcus-like organisms

The genera *Aerococcus*, *Gemella*, *Lactococcus*, *Leuconostoc*, and *Pediococcus* consist of organisms that resemble viridans streptococci. These bacteria have been isolated in clinical specimens and are associated with infections similar to those caused by enterococci and streptococci. These organisms are frequently identified when antimicrobial susceptibility testing of a "streptococcal" isolate reveals it to be vancomycin resistant. The vancomycin-resistant, gram-positive cocci are likely to be *Leuconostoc* or *Pediococcus*. *Aerococcus* is normally susceptible to vancomycin.

Abiotrophia and Granulicatella

Abiotrophia and *Granulicatella* spp., formerly known as the *nutritionally variant streptococci*, were first described in 1961. These bacteria grow as satellite colonies around other bacteria and require sulfhydryl compounds for growth. These organisms are part of the human oral and gastrointestinal microbiota. Most of the species are not groupable by the Lancefield system;

however, strains with group antigens A, F, H, L, and N have been reported. *Abiotrophia* and *Granulicatella* spp. are phenotypically related to members of the genera *Facklamia*, *Ignavigranum*, *Globicatella*, *Eremococcus*, *Dolosicoccus*, and *Aerococcus*.

Abiotrophia and *Granulicatella* spp. are a significant cause of bacteremia, endocarditis, and otitis media. *Granulicatella adiacens*, *Granulicatella elegans*, and *Granulicatella balaenopterae* have been isolated from blood cultures and tissue samples. Endocarditis resulting from these organisms is difficult to treat because of the increased tolerance of the organisms to antimicrobial agents. Surgery is usually required to achieve a cure. *Abiotrophia* and *Granulicatella* spp. have been linked to a few cases of osteomyelitis, endophthalmitis after cataract extraction, brain abscess, chronic sinusitis, septic arthritis, meningitis, and breast implant-associated infections.

Infection with *Abiotrophia* and *Granulicatella* spp. should be suspected when gram-positive cocci resembling streptococci are observed in positive blood cultures that subsequently fail to grow on subculture. It would be appropriate to use an SBA plate with a *S. aureus* streak and examine it for satellitism. Alternatively, media can be supplemented with 10 mg/L pyridoxal hydrochloride. *Granulicatella* spp. are often misdiagnosed and require a combination of approaches for identification. Tests for the production of β -glucuronidase, hippurate hydrolysis, arginine dihydrolase activity, and acid production from trehalose, sucrose, pullulan, and tagatose are used to differentiate the *Abiotrophia* and *Granulicatella* spp.

Aerococcus

Aerococcus is a common airborne organism that belongs to the family Aerococcaceae. It is a widespread, opportunistic pathogen causing an increasing number of infections associated with bacteremia, endocarditis, and UTI in immunocompromised patients. *Aerococcus viridans* has been linked to cases of bacteremia and endocarditis. *Aerococcus urinae* and *Aerococcus sanguinicola* have been associated with invasive diseases, such as sepsis, endocarditis, lymphadenitis, and peritonitis, often originating from the urinary tract. UTIs are seen more often in older adult women. However, invasive infections occur most frequently in older adult men, but the outcome is generally favorable. Both species are known to aggregate platelets and form biofilms.

Aerococcus resemble viridans streptococci on culture but are microscopically similar to staphylococci in that they occur as tetrads or clusters. These organisms sometimes show a weak catalase or pseudocatalase reaction. They grow in the presence of 6.5% NaCl and can easily be confused with enterococci. Their susceptibility pattern can also resemble that of the enterococci. Some strains of *A. viridans* are bile esculin positive and PYR positive. *A. urinae* is bile esculin negative and PYR negative. Because of their similarity to the staphylococci, streptococci, and enterococci, they are often difficult to accurately identify. However, 16S ribosomal ribonucleic acid (rRNA) sequencing and MALDI-TOF mass spectrometry testing seem to accurately identify *A. urinae*, *A. sanguinicola*, and *A. viridans*.

Dolosicoccus and Globicatella

Dolosicoccus and *Globicatella* are minor genera in the family Aerococcaceae. *D. paucivoran*, the single species in the genus, has been isolated in blood specimens. *D. paucivoran* can be

differentiated from *Globicatella* spp. based on biochemical and molecular tests.

Globicatella spp. were first described in 1992 and isolated from blood, urine, and CSF in patients with bacteremia, UTI, and meningitis. These facultative anaerobic, catalase-negative, α -hemolytic, gram-positive cocci are rarely isolated in clinical laboratories and often misidentified. The only species identified in human infection is *G. sanguinis*.

Dolosigranulum

Dolosigranulum spp. were first described in 1993 and are facultatively anaerobic, non motile cocci that occur in pairs, tetrads, or clusters. One species, *Dolosigranulum pigrum*, has been associated with eye infections, sepsis, healthcare-associated pneumonia, and ventilator-associated pneumonia in immunocompromised patients and patients with cystic fibrosis and COPD.

Facklamia

Facklamia species are α -hemolytic, catalase-negative, facultative anaerobic cocci seen in pairs or chains and have been misidentified as viridans streptococci. The species *F. hominis*, *F. ignava*, *F. sourekkii*, and *F. languida* have been isolated in human clinical specimens in patients with infective endocarditis and bacteremia.

Gemella

Gemella spp. are similar in colony morphology and habitat to viridans streptococci; they produce α -hemolysis or are nonhemolytic. The bacteria easily decolorize on Gram staining and often appear as gram-negative cocci in pairs, tetrads, clusters, or short chains. *Gemella* spp. have been isolated from cases of endocarditis, wounds, and abscesses. The most significant species is *Gemella haemolysans*.

Lactococcus

Lactococcus spp. are gram-positive cocci that occur singly, in pairs, or in chains and are physiologically similar to enterococci. On SBA, these organisms produce α -hemolysis or are nonhemolytic. These organisms were previously classified as group N streptococci. *Lactococcus* spp. have been isolated from patients with UTI and endocarditis. Production of acid from carbohydrates is useful in distinguishing *Lactococcus* spp. from enterococci. Also, these microorganisms do not react with the genetic probe in the AccuProbe *Enterococcus* culture confirmation test (Gen-Probe, San Diego, CA).

Leuconostoc

The genus *Leuconostoc* consists of catalase-negative, gram-positive microorganisms with irregular coccoid morphology (Fig. 15.12). These organisms share several phenotypic and biochemical characteristics with *Lactobacillus* spp., viridans streptococci (Fig. 15.13), *Pediococcus* spp., and *Enterococcus* spp. and are sometimes misidentified. Some species cross-react with the Lancefield group D antiserum. These microorganisms are intrinsically resistant to vancomycin. In nature, they are frequently found on plant surfaces and vegetables, and in milk products. They are recognized as opportunistic pathogens in

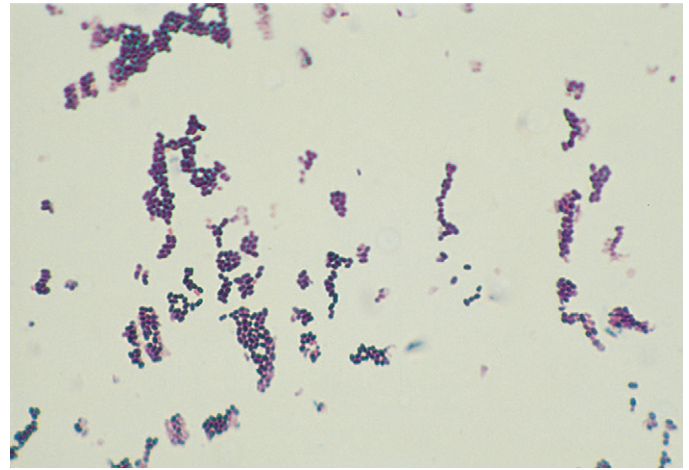


Fig. 15.12 Gram stain of *Leuconostoc* species ($\times 1000$).

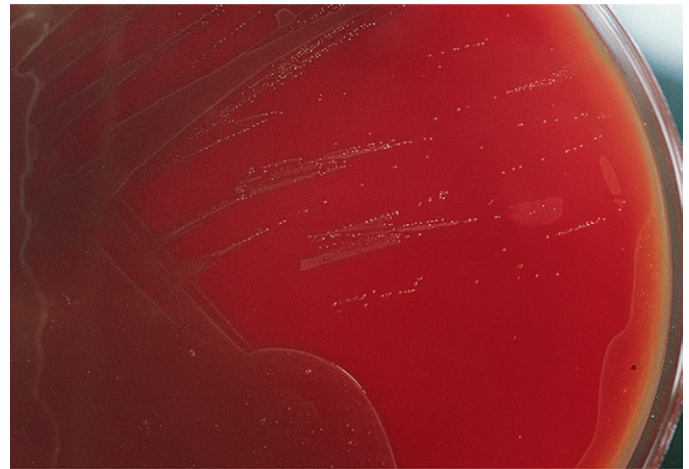


Fig. 15.13 Colonies of *Leuconostoc* species growing on sheep blood agar. *Leuconostoc* spp. may produce α -hemolysis and can resemble viridans streptococci.

patients who are immunocompromised or treated for underlying disease with vancomycin. These microorganisms have been isolated from cases of meningitis, bacteremia, UTIs, and pulmonary infections. Species associated with infection include *Leuconostoc citreum*, *Leuconostoc cremoris*, *Leuconostoc dextranicum*, *Leuconostoc lactis*, *Leuconostoc mesenteroides*, and *Leuconostoc pseudomesenteroides*. Biochemical identification is based on the absence of catalase, PYR, and LAP activities; hydrolysis of esculin in the presence of bile; growth in the presence of 6.5% NaCl; and production of gas from glucose.

Pediococcus

Members of the genus *Pediococcus* are facultatively anaerobic, gram-positive cocci (arranged in pairs, tetrads, and clusters) that can grow at 45° C. They may be misidentified as viridans streptococci or enterococci. Similar to *Leuconostoc* spp., *Pediococcus* spp. (*P. acidilactici*, *P. damnosus*, *P. dextrinicus*, *P. parvulus*, and *P. pentasaceus*) are intrinsically resistant to vancomycin. *Pediococcus* spp. have been associated with infections in patients who have underlying gastrointestinal abnormalities or who have previously undergone abdominal surgery. The organisms have also been linked to bacteremia, abscess

formation, and meningitis. Biochemical characteristics used to identify them include a positive bile esculin test, the presence of LAP activity, and absence of PYR activity. The organisms do not produce gas from glucose. Some of the strains are able to grow in the presence of 6.5% NaCl.

Vagococcus

Vagococcus spp. are commonly found in animals including pigs, cattle, horses, and cats. They are uncommonly found in humans but have been isolated from blood, cerebrospinal fluid, and wounds. The most common human isolate is *Vagococcus fluvialis*. Isolates typically express the Lancefield N antigen similar to *Lactococcus* and are difficult to identify and differentiate from *Enterococcus* spp. by conventional methods. *Vagococcus* spp. are facultative anaerobic, catalase-negative, motile, gram-positive cocci. MALDI-TOF mass spectrometry or 16s rRNA are the best methods for identification.

Laboratory diagnosis

Classification schemes

Several different approaches to the classification of catalase-negative, gram-positive cocci have been used. Four commonly used classification schemes are (1) hemolytic pattern on SBA; (2) physiologic characteristics; (3) serologic grouping or typing of C carbohydrate (Lancefield classification), capsular polysaccharide, or surface protein, such as the M protein of *S. pyogenes*; and (4) biochemical characteristics. The identification process for a streptococcal isolate in the clinical laboratory may use features from each scheme. More recently, MALDI-TOF mass spectrometry and genetic probes and sequencing have been used.

Hemolytic patterns

The medical laboratory scientist often makes an initial classification of the streptococci based on the hemolytic pattern of the isolate grown on SBA. Although hemolytic patterns can be helpful during the initial workup of an isolate, many species of streptococci show variable hemolytic patterns. The types of hemolysis possible are outlined in [Table 15.1](#).

Physiologic characteristics

The classification based on physiologic characteristics divides the streptococcal species into four groups: pyogenic streptococci, lactococci, enterococci, and viridans streptococci. Pyogenic streptococci produce pus; these organisms are mostly β -hemolytic and constitute most of the Lancefield groups. The lactococci are nonhemolytic organisms with a Lancefield group N antigen and are often found in dairy products. Enterococci comprise species found as part of the normal biota of the human intestine. Viridans streptococci are widely found as normal biota in the upper respiratory tract of humans. Most strains lack a C carbohydrate and are not part of the Lancefield classification; however, some have A, C, F, G, or N antigen. The viridans streptococci are α -hemolytic or nonhemolytic and are often seen as opportunistic pathogens. For the most part, this physiologic classification is historical.

Nevertheless, the terms *enterococci* and *viridans streptococci* remain and are still used to describe clinical isolates.

Lancefield classification scheme

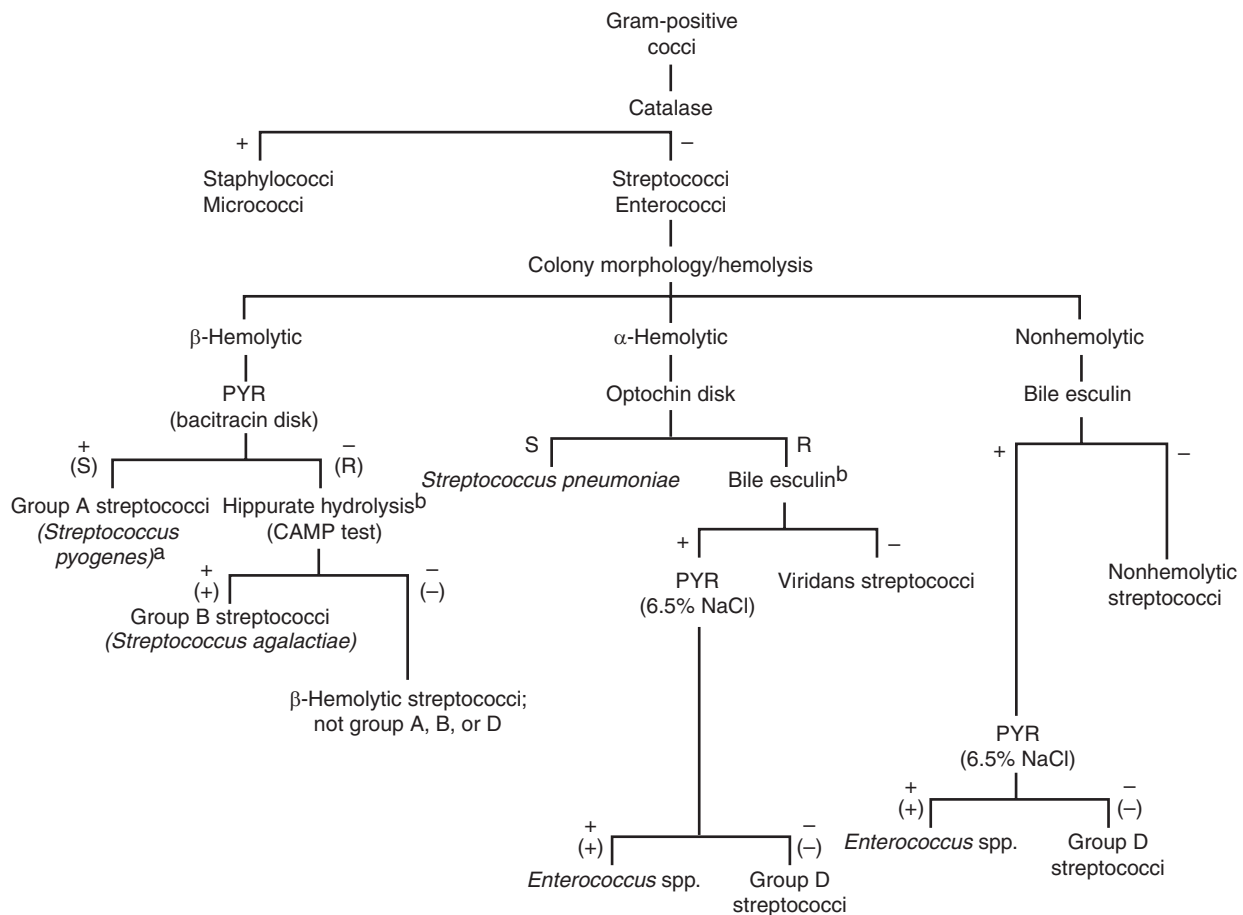
Because of readily available commercial kits, the **Lancefield classification system** is the most commonly used scheme. The classification system is based on extraction of C carbohydrate from the streptococcal cell wall by placing the organisms in dilute acid and heating for 10 minutes. The soluble antigen is used to immunize rabbits to obtain antisera to the various C carbohydrate groups. The Lancefield serologic grouping has been most significant in classifying and identifying β -hemolytic streptococci. However, because DNA relatedness has been applied to the classification and identification of α -hemolytic streptococcal species during the past several decades, investigators have found no correlation between genetic relationships and streptococcal group antigens. In contrast with group B β -hemolytic streptococci, in which only one species is identified, α -hemolytic streptococci as a whole are phenotypically and genotypically diverse and difficult to characterize.

The C carbohydrate is also present in streptococcal species other than those that produce β -hemolysis. Some are found as normal biota in animals or as animal pathogens, and others may be found in both humans and animals. These species belong to Lancefield groups A, B, C, D, F, G, and N, although not all members of these groups cause human infection. The classification of *Streptococcus* and *Enterococcus* spp. is shown in [Table 15.2](#).

Biochemical identification

Biochemical identification can be performed even by small laboratories. Although definitive identification requires a large number of biochemical characteristics or perhaps serologic methods, presumptive identification can be accomplished easily with a few key tests and characteristics ([Fig. 15.14](#)). Initial biochemical tests performed are often selected based on the hemolytic reaction of the isolate. In most cases, presumptive identification possesses a high enough rate of accuracy to be useful to the clinician and does not require the exhaustive additional tests that are needed to meet the criteria for definitive identification, especially for species in groups A, B, and D as well as *S. pneumoniae* and *Enterococcus* spp. However, speciation of the viridans streptococci does require a considerable increase in the number of tests.

The biochemical tests used for identification of streptococci include (1) bacitracin susceptibility, (2) the CAMP test, (3) hippurate hydrolysis, (4) PYR hydrolysis, (5) LAP, (6) VP, (7) β -D-glucuronidase, (8) bile esculin and salt tolerance, (9) optochin susceptibility, and (10) bile solubility tests. [Table 15.3](#) outlines the biochemical characteristics used for presumptive identification of selected members of the family Streptococcaceae and similar organisms. Several multitest commercial kits are available, such as the RapID STR (Thermo Fisher Scientific) shown in [Fig. 15.15](#). Some clinical laboratories forego biochemical testing and identify streptococci by detection of the group antigen. In selecting an identification scheme or kit, the medical laboratory scientist must evaluate the needs of the clinicians and patient population served, the cost of an expanded identification



^aRare isolates of enterococci are β -hemolytic. Enterococci are PYR positive and resistant to bacitracin.

^bPerform additional tests if isolate is from nonrespiratory source.

Fig. 15.14 Schematic diagram for the presumptive identification of gram-positive cocci. PYR, Pyrrolidonyl α -naphthylamide; R, resistant; S, susceptible.

scheme, the resources and abilities of the laboratory, and the usefulness of the data obtained. The procedures for many of the following tests can be found in [Appendix D](#) on Evolve.

Bacitracin susceptibility

Historically, bacitracin susceptibility has been used as an inexpensive test for presumptive identification of *S. pyogenes*. This method is helpful in screening for GAS in throat cultures. The throat swab is used to inoculate SBA containing SMZ, and a bacitracin disk is placed directly onto the agar. Growth of most interfering respiratory microbiota is inhibited by SMZ, but *S. pyogenes* and *S. agalactiae* grow. β -Hemolytic colonies that grow and are susceptible to bacitracin are presumptively identified as *S. pyogenes* (Fig. 15.16). *S. pyogenes* (group A) is susceptible to bacitracin and resistant to SMZ, whereas *S. agalactiae* (group B) is resistant to both bacitracin and SMZ. The PYR test, discussed later, is a more specific rapid test for GAS.

CAMP test

The **CAMP test** is used for presumptive identification of GBS. CAMP is an acronym derived from the first letters of the surnames of the individuals who first described the reaction: Christie, Atkins, and Munch-Petersen. The CAMP test can be performed in three ways. One is with the use of a β -lysin-producing strain of *S. aureus*; another is with the use of a disk impregnated with the β -lysin. Both methods take advantage

of the enhanced hemolysis occurring when the β -lysin and the hemolysin produced by GBS are in contact. *S. aureus* is inoculated in a straight line in the center of an SBA plate. The unknown streptococcal isolate is inoculated perpendicularly to the *S. aureus* inoculum. The result is a characteristic arrowhead-shaped hemolytic pattern (Fig. 15.17). When a disk containing β -lysin is used, the enhanced hemolysis is not a typical arrowhead shape because the disk is round. A third method, the rapid CAMP test (or spot CAMP test), involves placing a drop of extracted β -lysin on the area of confluent growth of the suspected GBS. After incubation at 35°C for at least 20 minutes, enhanced hemolysis is observed (Fig. 15.18).

Hippurate hydrolysis

A useful test for differentiating *S. agalactiae* from other β -hemolytic streptococci is the **hippurate hydrolysis test**. *S. agalactiae* possesses the enzyme hippuricase (also called *hippurate hydrolase*), which hydrolyzes sodium hippurate to form sodium benzoate and glycine. A 2-hour rapid test is available to detect the presence of hippurate hydrolase.

Pyrrolidonyl- α -naphthylamide hydrolysis

The PYR hydrolysis test detects the activity of L-pyrrolidonyl arylamidase, also called *pyrrolidonyl aminopeptidase*. *S. pyogenes* is the only species of *Streptococcus* that is PYR positive. Other genera that are PYR positive include *Enterococcus*, *Aerococcus*, and



RapID STR Color Guide

Test	Cavity	Positive Reactions	Negative Reactions	Comments
ARG	1	 	 	Without the addition of any reagents, read cavities 1-10 and record results: Cavity 1: Development of a red or dark orange color is a positive test; a yellow or yellow-orange color is a negative test.
ESC	2		  	Cavity 2: Development of a black color is a positive test; a clear, tan, or a light brown color is a negative test.
MNL	3			
SBL	4	 	 	Cavities 3-5: Development of a yellow or yellow-orange color is a positive test; a red or orange color is a negative test.
RAF	5			
INU	6	 		Cavity 6: Development of a yellow or orange color is a positive test; a red color is a negative test.
GAL	7			
GLU	8		 	Cavities 7-10: Development of a significant yellow color is a positive test; a very pale or clear color is a negative test.
NAG	9			
PO4	10			
TYR	7	 	  	Cavities 7-8: Development of a purple color, without regard to intensity, is a positive test; a clear, tan, or yellow color is a negative test.
HPR	8			
LYS	9	 	  	Cavities 9-10: Development of a very dark purple color is a positive test; a light to medium purple color is a negative test.
PYR	10			

Note: The RapID Color Guides are intended as an educational aid to be used in conjunction with the Technical Insert for the product. The reaction colors shown in the charts represent the typical shades of positive and negative colors.

800-447-3641 or 800-225-5443
(Technical Service)

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Fig. 15.15 RapID STR panel for the identification of *Streptococcus* spp. **A**, Commercial test well cartridge containing various substrates. **B**, Interpretive color guide to reactions. (Courtesy Thermo Fisher Scientific, Waltham, MA.)

Gemella. The PYR test takes advantage of the fact that *S. pyogenes* and *Enterococcus* spp. are able to hydrolyze the substrate PYR (Fig. 15.19). Several commercial systems are available. The specificity of this test for *Enterococcus* spp. is similar to that of the bile esculin and salt tolerance tests (see later). The PYR test is more specific for *S. pyogenes* than bacitracin susceptibility.

Leucine aminopeptidase

LAP is a peptidase that hydrolyzes peptide bonds adjacent to a free amino group. Because LAP reacts most quickly with leucine, it is called *leucine aminopeptidase*. The substrate, leucine- β -naphthylamide, is hydrolyzed to β -naphthylamine. After the addition of *p*-dimethylaminocinnamaldehyde reagent, a red color develops. Rapid commercial tests that use filter paper disks impregnated with the substrate are available. The LAP test is often used with the PYR test and is most

helpful in differentiating *Aerococcus* and *Leuconostoc* spp. from other gram-positive cocci. *Streptococcus*, *Enterococcus*, and *Pediococcus* spp. are LAP positive, and *Aerococcus* and *Leuconostoc* spp. are LAP negative.

Voges-Proskauer test

The **Voges-Proskauer (VP) test** is used to distinguish the small-colony-forming β -hemolytic anginosus group containing group A or C antigens from large-colony-forming pyogenic strains with the same antigens. Members of the anginosus group are positive. The VP test detects acetoin production from glucose. In the modified VP test, a heavy suspension of bacteria is made in 2 mL of VP broth. After about 6 hours of incubation at 35° C, a few drops of 5% α -naphthol and 40% potassium hydroxide are added. The tube is shaken vigorously to increase the concentration of dissolved oxygen,

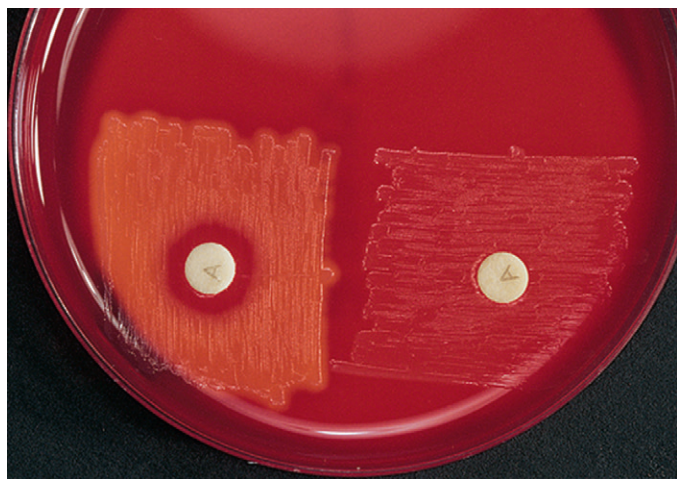


Fig. 15.16 Group A streptococci on sheep blood agar showing susceptibility to bacitracin. *Left*, Susceptible. *Right*, Resistant.

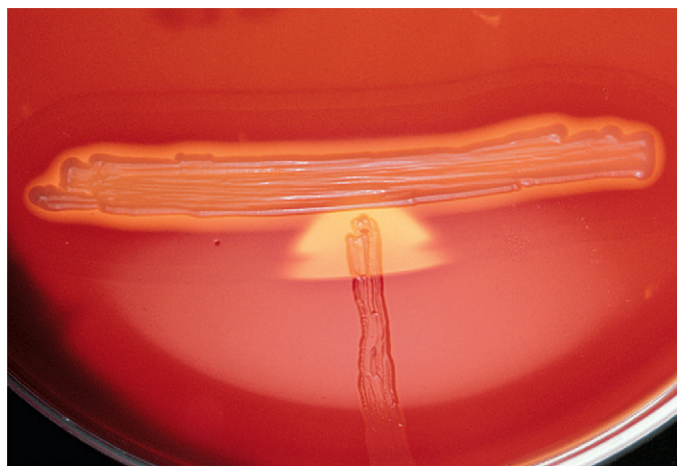


Fig. 15.17 Christie, Atkins, Munch-Petersen test for presumptive identification of group B streptococci. *Streptococcus agalactiae* shows the classic arrowhead shape near the streptococcal streak.



Fig. 15.18 Modification of the Christie, Atkins, Munch-Petersen test showing the enhanced hemolysis produced by *Streptococcus agalactiae* when a drop of extracted β -lysin is placed on the colony.

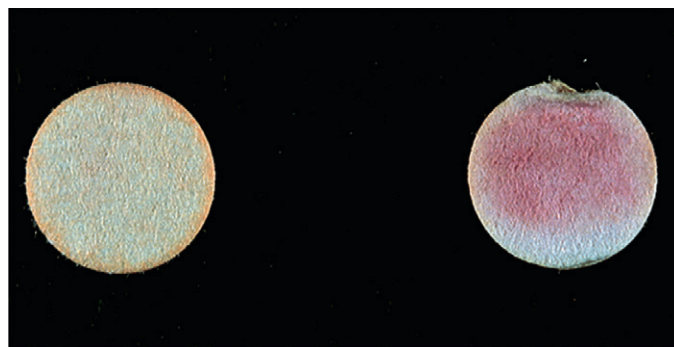


Fig. 15.19 Pyrrolidonyl- α -naphthylamide test for identifying *Streptococcus pyogenes* and *Enterococcus*. *Left*, Negative test. *Right*, Positive test.

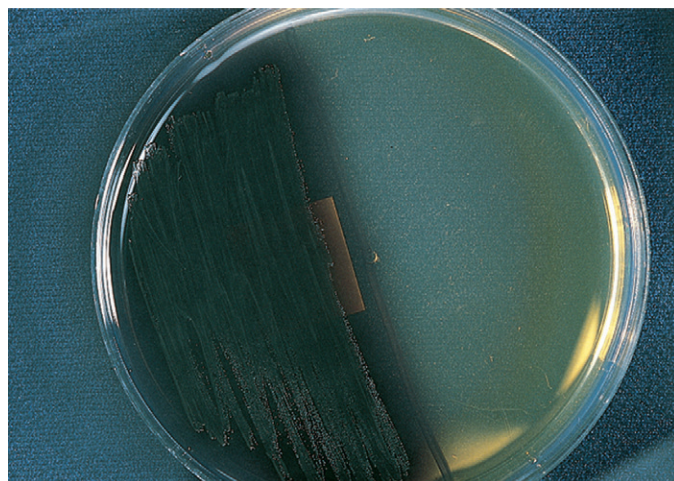


Fig. 15.20 Bile esculin test. *Left*, Positive test shows blackening of the agar. *Right*, Negative test.

and the broth is incubated at room temperature for 30 minutes. The formation of a red or pink color is a positive reaction.

β -D-glucuronidase

The β -D-glucuronidase test detects the action of β -D-glucuronidase, an enzyme found in isolates of large-colony-forming β -hemolytic group C and G streptococci but not in the small-colony-forming β -hemolytic *S. anginosus* group. Several commercially prepared rapid assays are available. A fluorogenic assay using methylumbelliferyl- β -D-glucuronide has also been described.

Bile esculin and salt tolerance

The bile esculin hydrolysis test and the salt tolerance test have been mainstays in identification schemes for nonhemolytic, catalase-negative, gram-positive cocci. The bile esculin test is a two-step test detecting growth of bacteria in the presence of 40% bile and the ability to hydrolyze esculin. Group D streptococci and *Enterococcus* spp. test positive (Fig. 15.20). Organisms positive for bile esculin are separated into group D streptococci or *Enterococcus* by the salt tolerance test. Growth in 6.5% NaCl broth is used to identify *Enterococcus* and *Aerococcus* organisms. Some species of *Pediococcus* and *Leuconostoc* grow in 6.5% NaCl broth when incubated for 24 hours. However, group D streptococci do not grow in a 6.5% NaCl broth.

Optochin susceptibility

In the **optochin test**, a filter paper disk containing optochin (ethylhydrocuprein hydrochloride) is added to the surface of an SBA plate that has just been inoculated with an α -hemolytic *Streptococcus*. The plate is incubated overnight at 35° C in a CO₂ incubator. A zone of inhibition greater than 14 mm with a 6-mm disk or a zone of inhibition greater than 16 mm with a 10-mm disk is considered susceptible and a presumptive identification of *S. pneumoniae* (Fig. 15.21). Isolates producing smaller zones should be tested for bile solubility to confirm their identity.

Bile solubility

The **bile solubility** test correlates well with optochin susceptibility. The test for bile solubility takes advantage of the *S. pneumoniae* autocatalytic enzyme amidase. Under the influence of a bile salt or detergent, the organism's cell wall lyses during cell division. A suspension of *S. pneumoniae* in a solution of sodium deoxycholate lyses, and the solution becomes clear. Other α -hemolytic organisms do not undergo autolysis, and the solution remains cloudy. Suspensions of bacteria made in saline serve as negative controls.

Noncultural identification

Immunoassays

Identification of streptococci, particularly GAS, can be accomplished by the detection of the group-specific antigen from either isolated colonies or, in some cases, a direct clinical specimen such as a throat swab. Isolated colonies can be identified by extracting the C carbohydrate by means of acid or heat. The extract containing the specific group carbohydrate is used in a capillary precipitin test or a slide agglutination test. In the capillary precipitin test, antiserum to the specific group carbohydrate is overlaid with the solution containing the streptococcal extract. After 5 to 10 minutes, the interface between the extraction solution and the antiserum is examined carefully for a white precipitate, which indicates a positive reaction. Each extract can be tested with numerous antisera specific to the various group antigens.

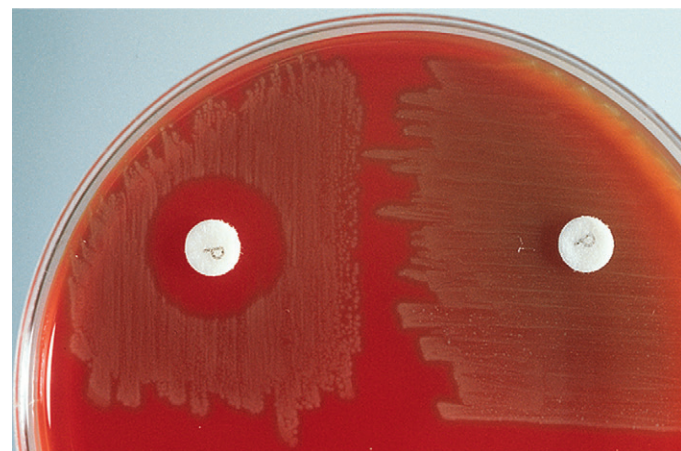


Fig. 15.21 Optochin susceptibility test on sheep blood agar. *Left*, *Streptococcus pneumoniae* showing susceptibility to optochin. *Right*, Resistant viridans streptococci.

An alternative method that is easier to perform is the slide agglutination test, which uses a carrier particle, such as latex, for the group-specific antibody. When an antibody-antigen reaction occurs, it is seen macroscopically as agglutination of the particles (Fig. 15.22). These immunoassays provide a definitive identification of the Lancefield group to which the isolate belongs; however, when the group comprises several species, the methods do not give a species identification. If the results indicate that the isolate belongs to group B, it can be identified as *S. agalactiae*. The cost of these methods is higher than that of the standard culture approaches; however, the results are often obtained more quickly and are often more accurate.

More than two dozen different antigen-detection products are commercially available, many of which use latex agglutination, an optical immunoassay, or an enzyme-linked immunosorbent assay system to detect GAS from throat swabs. These are designed primarily for use in a clinic or physician's office. Although a positive finding allows the primary care provider to treat *S. pyogenes* infections without waiting for culture results, a negative result in children or adolescents necessitates a throat culture because the sensitivity of direct detection methods for GAS is not high enough to ensure that the negative result is not a false-negative. The positive predictive value in adult populations is low. Additionally, the cost of using direct detection methods can be significantly higher than that of culture only. The direct detection method for GAS can be a valuable diagnostic tool in the right context; however, the cost and sensitivity must be weighed carefully.

Nucleic acid probes

Nucleic acid probe assays are commercially available and offer rapid results and increased specificity compared with conventional identification methods. However, nucleic acid probe assays cost more. The LightCycler (Roche Applied Science, Indianapolis, IN) is a real-time PCR instrument that can identify GAS and GBS as well as groups C and G. The assay detects the *cfb* gene, which encodes the CAMP-factor protein of GBS and the *ptsI* (phosphotransferase) gene of GAS. Groups C and G have a *ptsI* gene sequence similar to that of GAS, but the instrument is able to distinguish among the three



Fig. 15.22 Slide agglutination test for grouping streptococci.

groups. Compared with bacterial throat cultures for GAS, the LightCycler has a sensitivity of 93% and a specificity of 98%.

The AccuProbe pneumococcus test (Gen-Probe, San Diego, CA) uses a DNA probe to detect the 16S rRNA sequence of *S. pneumoniae*. The less than 1% difference in the rRNA sequence between *S. pneumoniae* and *S. oralis* and *S. mitis* raises questions about the specificity of this assay. The Smart Cycler (Cepheid, Sunnyvale, CA) real-time PCR instrument also provides kits for the detection of streptococci. This instrument has four-channel optics, allowing the detection of several targets in one sample (multiplex).

Susceptibility testing

Antimicrobial susceptibility testing for pathogens is used to guide therapy and identify resistance of the pathogen to antimicrobial agents used for therapy. In the case of *S. pneumoniae* and other streptococci, the Clinical and Laboratory Standards Institute (CLSI) has established procedures for both disk diffusion and broth dilution assays. Commercial methods including the Etest (AB Biodisk, Solna, Sweden) are also available. Elevated MICs may be observed with the Etest for some antimicrobial agents, possibly resulting from CO₂ incubation conditions. Testing can also be performed using automated instruments such as the BD Phoenix (Becton Dickinson, Franklin Lakes, NJ), MicroScan (Siemens Healthcare Diagnostics, Tarrytown, NY), and Vitek (bioMérieux) systems.

Penicillin remains the drug of choice in treating most streptococcal infections. However, penicillin-resistant *S. pneumoniae* and viridans group isolates have been reported worldwide, and the incidence of penicillin-resistant *S. pneumoniae* seems to be increasing. Resistance of *S. pneumoniae* to penicillin and other β -lactams occurs by alteration in the cell wall penicillin-binding proteins. Although pneumococcal resistance to β -lactams is common, high-dose parenteral penicillin G and many other parenteral and oral β -lactams have been effective in treatment. The current penicillin susceptibility breakpoint of 2 mg/mL for pneumococcal infection and 0.06 mg/mL for meningitis was published by the CLSI based on microbiological, pharmacokinetic/pharmacodynamic, and clinical information. Erythromycin and narrow-spectrum cephalosporins are alternatives, although erythromycin resistance encoded by the *erm* and *mef* genes has been reported. Multidrug-resistant pneumococci have also been reported.

Vancomycin is an effective antimicrobial for treating infections caused by gram-positive organisms. Gram-positive isolates are often routinely tested for vancomycin susceptibility. Table 15.3 lists the usual patterns of vancomycin susceptibility for many gram-positive cocci. In particular, *Pedococcus* and *Leuconostoc* are resistant to vancomycin. *E. faecalis* and *E. faecium* have emerged as therapeutically challenging organisms because of increasing resistance to vancomycin and penicillin. More recently, these VRE isolates also have a high level of resistance to aminoglycosides. The combination of an aminoglycoside with a cell wall-active agent, such as ampicillin, penicillin, or vancomycin, is often required for the treatment of enterococcal infection. The combination is effective in the absence of high-level aminoglycoside resistance. The CLSI recommends screening of enterococci isolated from blood cultures or specimens such as heart valve tissue for high-level aminoglycoside resistance with both gentamicin and streptomycin. Real-time PCR assays, such as the BD GeneOhm VanR

(BD Diagnostic Systems) and Xpert vanA/vanB (Cepheid) assays, can be used for rapid detection of VRE carrying the *vanA* and *vanB* genes from rectal and perianal swabs.

POINTS TO REMEMBER

- The organisms included in the family Streptococcaceae and the *Streptococcus*-like organisms are catalase-negative, gram-positive cocci usually arranged in pairs or chains.
- Hemolysis on SBA is often a starting point for the identification of streptococci and similar organisms.
- Many streptococci can be categorized based on Lancefield group antigens.
- Key tests for the identification of streptococci and similar organisms include bacitracin susceptibility, CAMP, hippurate hydrolysis, pyrrolidonyl- α -naphthylamide (PYR) hydrolysis, leucine aminopeptidase (LAP), Voges-Proskauer (VP), β -D-glucuronidase, bile esculin, salt tolerance, optochin susceptibility, and bile solubility tests.
- Many clinical microbiology laboratories use group antigen detection to identify the streptococci.
- Group A streptococci are important pathogens that cause acute bacterial pharyngitis, skin and soft tissue infections, and invasive infections, including necrotizing fasciitis.
- Emerging resistance of *Streptococcus pneumoniae* to numerous antimicrobials is becoming a major health concern.
- Viridans streptococci, although usually nonpathogenic, can cause bacteremias, abscesses, and oral infections such as gingivitis and dental caries.
- Nutritionally variant streptococci are classified within the genera *Granulicatella* and *Abiotrophia*.
- Enterococci cause approximately 5% to 10% of infections in patients with bacterial endocarditis.
- *Streptococcus agalactiae* is a significant cause of invasive disease in newborns.
- Group C and G streptococci are important human pathogens associated with infections such as endocarditis, meningitis, primary bacteremia, necrotizing fasciitis, and myositis.

LEARNING ASSESSMENT QUESTIONS

1. Name three tests that could be performed to aid in the identification of *Streptococcus pyogenes*.
2. A β -hemolytic, catalase-negative, gram-positive coccus is found to be resistant to bacitracin and sulfamethoxazole. Which of the following is a likely presumptive identification?
 - a. Group A streptococci
 - b. Group B streptococci
 - c. Group D streptococci
 - d. Enterococci
3. The Christie, Atkins, Munch-Petersen (CAMP) test is based on enhanced hemolysis between CAMP factor and β -lysin from which of the following?
 - a. *Streptococcus agalactiae*
 - b. *Staphylococcus epidermidis*
 - c. *Staphylococcus aureus*
 - d. *Enterococcus*

4. A nonhemolytic, catalase-negative, gram-positive coccus is pyrrolidonyl α -naphthylamide (PYR) positive. You should also expect the isolate to have which of the following characteristics?
 - a. Bile esculin positive
 - b. Salt (6.5%) tolerant
 - c. Bile soluble
 - d. Both a and b
5. The optochin test is most valuable in the identification of which of the following?
 - a. α -Hemolytic streptococci
 - b. β -Hemolytic streptococci
 - c. Nonhemolytic streptococci
 - d. Both a and b
6. Which antimicrobial agent or agents are most commonly used to treat infections caused by pyogenic streptococci?
7. *Streptococcus pyogenes* has been associated with which invasive infections?
8. Which streptococcal species is the most common cause of community-acquired bacterial pneumonia?
9. What is the clinical significance of group B streptococci isolated from a vaginal culture of a pregnant woman?
10. How would you recover nutritionally variant streptococci from clinical samples such as blood?
11. A 9-year-old boy complains of fever, severe headache, and sore throat. On examination, his pharynx is red, and his tonsils are swollen. The throat culture grows β -hemolytic colonies on sheep blood agar and is identified as group A streptococci. Which two serious complications can result if the infection is not treated (post-infection sequelae)?
12. You isolated α -hemolytic colonies from the sputum sample of a 75-year-old patient. When tested, the colonies were found to be catalase negative, optochin (P disk) resistant, bile solubility test negative, and bile esculin test negative. This isolate can be identified as which of the following?
 - a. *Streptococcus agalactiae*
 - b. Viridans streptococci
 - c. *Streptococcus pneumoniae*
 - d. *Enterococcus faecalis*
13. A cerebrospinal fluid sample from a neonate grows gram-positive cocci that are β -hemolytic and catalase negative. Which of the following group of tests would be most helpful in identifying the organism?
 - a. Bacitracin, CAMP, and hippurate hydrolysis
 - b. Optochin, CAMP, and bile solubility
 - c. Growth in 6.5% NaCl and bile esculin
 - d. Gram stain and motility
14. Which of the following organisms would be best described as a gram-positive coccus that is a normal colonizer of the gastrointestinal (GI) tract that enters the bloodstream causing bacteremia and is thought to be closely associated with GI disorders including colon cancer?
 - a. Group B streptococci
 - b. Viridans streptococci
 - c. Group D streptococci
 - d. *Staphylococcus aureus*
15. Streptolysin S and streptolysin O are virulence factors produced by which of the following organisms?
 - a. Group B streptococci
 - b. Viridans streptococci
 - c. Group D streptococci
 - d. *Streptococcus pyogenes*

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Aerobic gram-positive bacilli

Steven D. Mahlen and Amanda T. Harrington

CHAPTER OUTLINE

Non-spore-forming, nonbranching, catalase-positive bacilli, 348

- Corynebacterium*, 348
- Other corynebacteria, 351
- Identification of coryneform bacteria, 354
- Rothia*, 354
- Related genera, 354

Non-spore-forming, nonbranching, catalase-negative bacilli, 356

- Erysipelothrix rhusiopathiae*, 356
- Arcanobacterium* and *Trueperella*, 358
- Gardnerella vaginalis*, 358

Non-spore-forming, branching, aerobic actinomycetes, 360

- Nocardia*, 360
- Other actinomycetes, 362

Spore-forming, nonbranching, catalase-positive bacilli, 364

- Bacillus*, 364

Bibliography, 370

OBJECTIVES

After reading and studying this chapter, you should be able to:

1. Compare the general characteristics of the aerobic gram-positive bacilli discussed in this chapter.
2. Compare the clinical significance of the aerobic gram-positive bacilli and how the infections they cause are acquired.
3. Describe the types of clinical specimens that are likely to contain the aerobic gram-positive bacilli discussed in this chapter.
4. Describe the microscopic morphology and colony appearance of the aerobic gram-positive bacilli discussed in this chapter.
5. Describe the clinical infections associated with *Corynebacterium diphtheriae*.
6. Justify the use of in vitro diphtheria exotoxin detection by *C. diphtheriae*.
7. Discuss several nondiphtheria *Corynebacterium* spp. that are capable of causing clinical infection in humans.

8. Compare the culture and identifying characteristics of *Listeria monocytogenes* with *Streptococcus agalactiae* (group B streptococci).
9. Differentiate *L. monocytogenes* from other non-spore-forming gram-positive bacilli and streptococci.
10. Differentiate *Erysipelothrix rhusiopathiae* from other non-spore-forming gram-positive bacilli.
11. Differentiate *Arcanobacterium haemolyticum* from other non-spore-forming gram-positive bacilli and β -hemolytic streptococci.
12. Differentiate *Gardnerella vaginalis* from other non-spore-forming gram-positive bacilli on the basis of pathogenesis and laboratory identification.
13. Justify using a Gram stain of vaginal discharge instead of culture to diagnose bacterial vaginosis.
14. Describe the clinical infections associated with *Nocardia*, *Actinomadura*, and *Streptomyces*.
15. Differentiate infections caused by *Nocardia*, *Actinomadura*, *Streptomyces*, *Gordonia*, and *Rhodococcus* from infections caused by fungal agents.
16. Describe *Tropheryma whippelii* and Whipple disease.
17. Compare the appearance of bacterial spores when visualized with Gram stain and spore stain.
18. Describe the clinical infections associated with *Bacillus anthracis*.
19. Compare the relationship among the three exotoxin proteins of *B. anthracis*.
20. Describe the differential tests used to identify *B. anthracis*.
21. Discuss the clinical significance of *Bacillus* spp. other than *B. anthracis*.
22. Describe the clinical significance of *Bacillus cereus*.
23. Differentiate *B. anthracis* from other *Bacillus* spp.

KEY TERMS

Actinomycotic mycetoma

Anthrax

Babès-Ernst granules

Bacterial vaginosis (BV)

Cold enrichment

Cystine-tellurite blood agar (CTBA)

Diphtheria

Diphtheria toxin

Edema factor (EF)

Elek test

Eschar**Eumycotic mycetoma****Human blood bilayer Tween (HBT) agar****Lethal factor (LF)****Loeffler medium****Medusa head****Pleomorphic****Protective antigen (PA)****Sulfur granules****Tumbling motility****Whipple disease****Woolsorter disease****Case in point**

A 76-year-old female patient who was receiving corticosteroid therapy for a malignant tumor complained to her physician of fever and headache of 7 days' duration. Her headache had become progressively worse, and her temperature was elevated. A complete blood count was performed and showed a slightly elevated white blood cell (WBC) count with normal distribution. A lumbar puncture was performed, and the following laboratory results were obtained from the cerebrospinal fluid (CSF):

- 250 WBC/mL
- Glucose 30 mg/dL (serum glucose was 105 mg/dL)
- Protein 180 mg/dL

No bacteria were observed on a Gram-stained smear of the CSF. The CSF was inoculated onto sheep blood agar (SBA) and chocolate agar. β -Hemolytic colonies grew on the SBA 2 days later. Similar colony growth was present on the chocolate agar. Gram stain morphology revealed a pleomorphic, gram-positive, non-spore-forming bacillus. The isolate had the following biochemical characteristics:

- Catalase positive
- Esculin hydrolysis positive
- Hippurate hydrolysis positive
- Motile at room temperature, but not at 35° C
- Christie, Atkins, Munch-Peterson (CAMP) test positive (block, not arrow shaped)

Issues to consider

After reading the patient's case history, consider:

- Key tests to be performed to differentiate non-spore-forming gram-positive bacilli
- Factors that increase an individual's risk for infection by non-spore-forming gram-positive bacilli
- The distribution in nature of non-spore-forming gram-positive bacilli and the species that constitute the normal bacterial biota of humans

This chapter discusses a large diverse group of bacteria commonly encountered in the clinical microbiology laboratory. Most species are found in the environment and are easily isolated from water and soil. Most of these organisms are not highly pathogenic but are being isolated from clinical infections with increasing frequency. Bacteria that belong to the aerobic, gram-positive bacilli group include the spore-forming genus *Bacillus*; non-spore-forming bacteria, including the genera *Corynebacterium*, *Arcanobacterium*, *Rhodococcus*, *Listeria*, *Erysipelothrix*, and *Gardnerella*; and branching, non-spore-forming aerobic actinomycetes, including *Nocardia*. Fig. 16.1 shows a schematic diagram for the presumptive identification of aerobic, gram-positive bacteria. Members of the genus *Lactobacillus* are non-spore-forming gram-positive rods, which are frequently isolated from urogenital specimens from women and are incubated aerobically. However,

these organisms are aerotolerant anaerobes and are discussed in Chapter 22.

A wide range of clinical conditions results from infection with these organisms. Although several of these organisms are frequently isolated in the clinical laboratory, they are typically considered contaminants or commensals (e.g., *Bacillus* and *Corynebacterium*). Several of these organisms are rarely encountered but cause significant disease (*Listeria*, *Erysipelothrix*, *Corynebacterium diphtheriae*, and *Bacillus anthracis*). For some species, the frequency of isolation is increasing, and new clinical syndromes are being established, especially in immunocompromised patients. As genetic and molecular biology tools progress, the diversity of many of these genera is being identified (e.g., *Nocardia*, *Corynebacterium*), and new clinical correlations are being made.

Non-spore-forming, nonbranching, catalase-positive bacilli

Corynebacterium

General characteristics

The genus *Corynebacterium* is a large diverse group of bacteria that includes animal and human pathogens as well as saprophytes and plant pathogens. There are more than 200 species in the genus. Most of the species are found as normal microbiota on the skin and mucous membranes of humans and animals, and some species are found worldwide in the environment. On the basis of 16S ribosomal ribonucleic acid (rRNA) sequencing, corynebacteria are closely related to mycobacteria and nocardiae. *Corynebacterium* can be divided into nonlipophilic and lipophilic species. Lipophilic corynebacteria are often considered fastidious and grow slowly on standard culture media; cultures often must be incubated for at least 48 hours before growth is detected. However, growth is enhanced if lipids are included in the culture medium.

On Gram stain, corynebacteria are slightly curved, gram-positive bacilli with nonparallel sides and slightly wider ends, producing the described "club shape" or coryneform. The term *diphtheroid*, meaning "diphtheria-like," is sometimes used in reference to this microscopic morphology for nonpathogenic species. The classification of corynebacteria continues to evolve. There is a low rate of accurate species-level identification for many clinical isolates when using traditional biochemical methods of identification, and it is not possible to identify a significant proportion of coryneform-like isolates to the species level without matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry or 16S DNA gene sequencing.

The most significant pathogen of the group, *C. diphtheriae*, has been extensively studied and is well characterized. Disease caused by *C. diphtheriae* is referred to as **diphtheria**. Nondiphtheria *Corynebacterium* spp. are frequently isolated from clinical specimens, and they are often dismissed as commensal microbiota. However, similar to many organisms previously thought to be commensal nonpathogenic organisms, *Corynebacterium* spp. isolated from various body sites are opportunistic pathogens, especially in immunocompromised patients. Nondiphtheria *Corynebacterium* spp. that

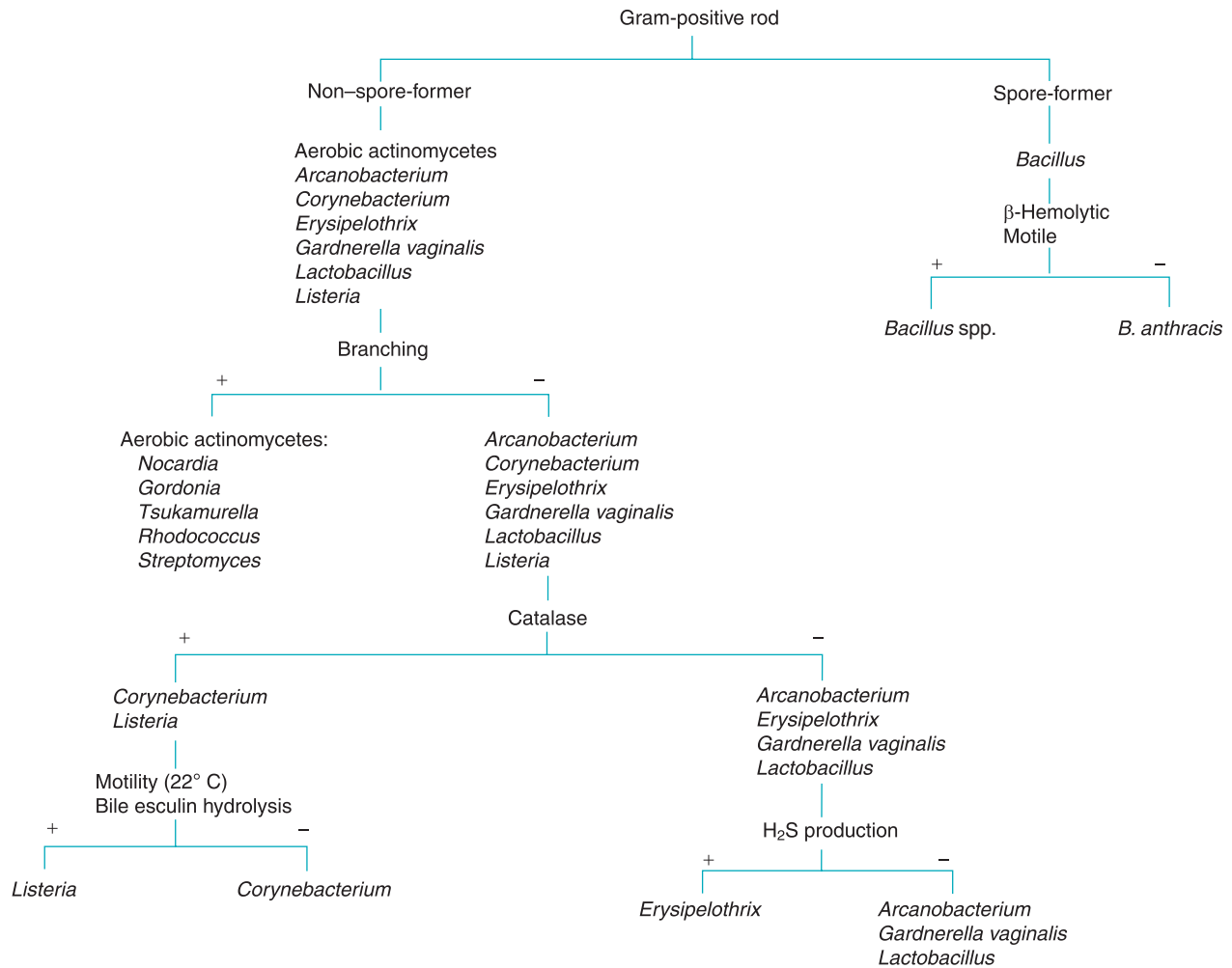


Fig. 16.1 Schematic diagram for the identification of Gram-positive bacteria. *Lactobacillus* is an aerotolerant anaerobe discussed in Chapter 22. H_2S , Hydrogen sulfide.

produce disease in humans include, but are not limited to, *Corynebacterium amycolatum*, *Corynebacterium pseudodiphtheriticum*, *Corynebacterium pseudotuberculosis*, *Corynebacterium jeikeium*, *Corynebacterium striatum*, *Corynebacterium ulcerans*, and *Corynebacterium urealyticum*.

Corynebacterium diphtheriae

Virulence factors

Diphtheria toxin is the major virulence factor associated with *C. diphtheriae*. This exotoxin is produced by strains of *C. diphtheriae* infected with a lysogenic corynebacteriophage that carries the *tox* gene for diphtheria toxin. Nontoxic strains can be converted to *tox* positive by infection with the appropriate corynebacteriophage. Toxin-producing *C. diphtheriae* causes diphtheria; however, *C. belfantii*, *C. ulcerans*, and *C. pseudotuberculosis* are part of a larger complex that can carry and produce the toxin.

Diphtheria toxin is a protein of 62,000 daltons (Da). It is composed of two fragments: A and B. The toxin is exceedingly potent and is lethal for humans in amounts of 100 ng/kg body weight. The toxicity is caused by the ability of diphtheria toxin to block protein synthesis in eukaryotic cells. The toxin is secreted by the bacterial cell and is nontoxic until exposed to

trypsin. Trypsinization cleaves the toxin into the two fragments, which are held together by a disulfide bridge. Both fragments are necessary for cytotoxicity. Fragment A is responsible for the cytotoxicity, and fragment B binds to receptors on human cells and mediates the entry of fragment A into the cytoplasm. On reaching the cytoplasm, fragment A disrupts protein synthesis. Fragment A splits nicotinamide adenosine dinucleotide to form nicotinamide and adenosine diphosphoribose (ADPR). ADPR binds to and inactivates **elongation factor 2 (EF-2), an enzyme required for elongation of polypeptide chains on ribosomes**. Inactivation of EF-2 inhibits protein synthesis.

Production of the toxin in vitro depends on numerous environmental conditions, including an **alkaline pH (7.8 to 8.0)**, oxygen, and, **most importantly, the iron concentration in the environment**. The amount of iron needed for optimal toxin production is less than the amount needed for optimal growth. The toxin is released in significant amounts only when the available iron in the culture medium is exhausted.

Clinical infections

C. diphtheriae causes two different forms of disease in humans: **respiratory and cutaneous diphtheria**. Respiratory diphtheria is found worldwide but is uncommon in North America and western Europe because of widespread **vaccination**.

In the United States since universal vaccination began in the 1940s, cases occur most often in nonimmunized populations. Between 2004 and 2019, only five probable or confirmed diphtheria cases were reported. However, the number of diphtheria cases reported globally has been increasing gradually. The World Health Organization estimates that less than 16,000 cases were reported in 2018, which is more than double the yearly average for the prior decade. The U.S. Centers for Disease Control and Prevention (CDC) recommends that international travelers ensure that they are up to date on all vaccinations. Individuals who were vaccinated as children and have not been revaccinated as adults are susceptible to infection.

Humans are the only natural hosts for *C. diphtheriae*. Both toxigenic and nontoxigenic strains can cause infection; however, disease caused by nontoxigenic strains is typically less severe and less invasive. The bacteria are carried in the upper respiratory tract and spread by **droplet or hand-to-mouth contact**. The incubation period averages 2 to 5 days. The illness begins gradually and is characterized by low-grade fever, malaise, and a mild sore throat. The most common site of infection is the tonsils or the pharynx. The organisms rapidly multiply on the epithelial cells, and the toxigenic strains of *C. diphtheriae* produce toxin locally, causing tissue necrosis and exudate formation and triggering an inflammatory reaction. This combination of cell necrosis and exudate forms a tough gray-to-white pseudomembrane that attaches to the tissues. It may appear on the tonsils and then spread downward into the larynx and the trachea. There is the potential for suffocation if the membrane blocks the air passage or if it is dislodged, perhaps as the result of sampling for a throat culture.

The toxin is also absorbed and can produce various systemic effects involving the **kidneys, heart, and nervous system**, although most cells possess the receptor for the toxin and may be affected. Death often is a result of cardiac failure. Another effect of the toxin is a demyelinating peripheral neuritis, which can result in paralysis following the acute illness.

Other nonrespiratory sites may be infected, although much less often compared with the upper respiratory tract. In the cutaneous form of diphtheria, which is prevalent in the tropical regions of the world, the toxin also is absorbed systemically, but systemic complications are less common than those of upper respiratory tract infections. Cutaneous diphtheria caused by toxigenic strains consists of nonhealing ulcers with a dirty gray membrane. Although this form is unusual in the United States, there have been outbreaks caused by close person-to-person contact. When isolated in culture from cutaneous disease, *C. diphtheriae* is often recovered along with ***Streptococcus pyogenes* and *Staphylococcus aureus***. In addition, with the adoption of MALDI-TOF mass spectrometry in clinical microbiology laboratories, there has been an increased rate of reporting for *C. diphtheriae* from cutaneous samples.

An effective toxoid vaccine—formalin-treated diphtheria toxin—is available. It is part of the trivalent diphtheria, tetanus, and pertussis (acellular pertussis for pediatric formulations) vaccine. Children receive a series of five injections beginning at 2 months of age, with the fifth administered between 4 and 6 years of age. A combination diphtheria-tetanus vaccine is recommended every 10 years thereafter. The

vaccine induces antibody to diphtheria toxin, preventing disease but not infection by *C. diphtheriae*.

Laboratory diagnosis

Microscopy

C. diphtheriae is a highly **pleomorphic (many shapes) gram-positive bacillus that appears in palisades (cells lie in parallel rows) or as individual cells lying at sharp angles to each other in V and L formations**. Although they may be demonstrated by other *Corynebacterium* spp., club-shaped swellings and beaded forms are common (Fig. 16.2). The organisms often stain irregularly, especially when stained with methylene blue, giving them a beaded appearance. The metachromatic areas of the cell, which stain more intensely than other parts, are called **Babès-Ernst granules**. They represent accumulation of polymerized polyphosphates. The presence of Babès-Ernst granules indicates the accumulation of nutrient reserves and differs with the type of medium and the metabolic state of the individual cells.

Culture characteristics

C. diphtheriae is a facultative anaerobe. It grows best under aerobic conditions and has an optimal growth temperature of 37° C, although multiplication occurs within the range of 15° to 40° C. Although *C. diphtheriae* grows on nutrient agar, better growth is usually obtained on a medium containing blood or serum, such as **Loeffler serum or Pai agars**. Characteristic microscopic morphology is demonstrated well when organisms are grown on **Loeffler medium**. On SBA (Fig. 16.3), the organism can have a very small zone of β -hemolysis.

Cystine-tellurite blood agar (CTBA), a modification of Tinsdale medium, contains sheep red blood cells, bovine serum, cystine, and potassium tellurite. CTBA is both a selective medium and a differential medium. The potassium tellurite inhibits many noncoryneform bacteria. When grown on CTBA, corynebacteria form black or brownish colonies from the reduction of tellurite. However, this appearance is not unique for *C. diphtheriae*, so care must be taken not to presumptively identify other genera that produce black colonies (e.g., *Staphylococcus* and *Streptococcus*) as *Corynebacterium*.

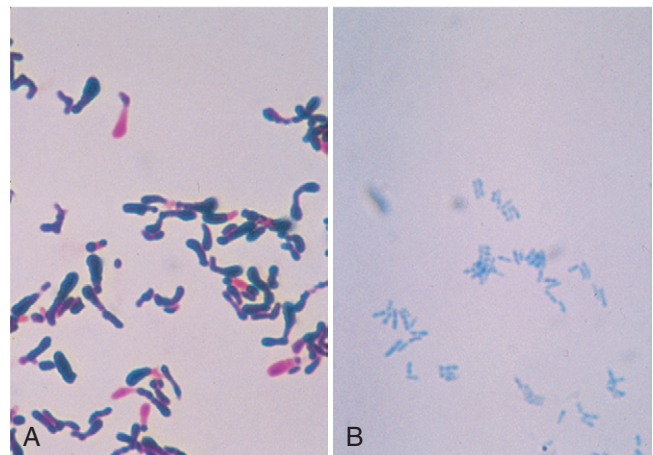


Fig. 16.2 **A**, Microscopic Gram stain of diphtheroids (original magnification $\times 1000$). **B**, Microscopic Loeffler methylene blue stain of *Corynebacterium* spp. ($\times 1000$). (Courtesy Cathy Bissonette.)

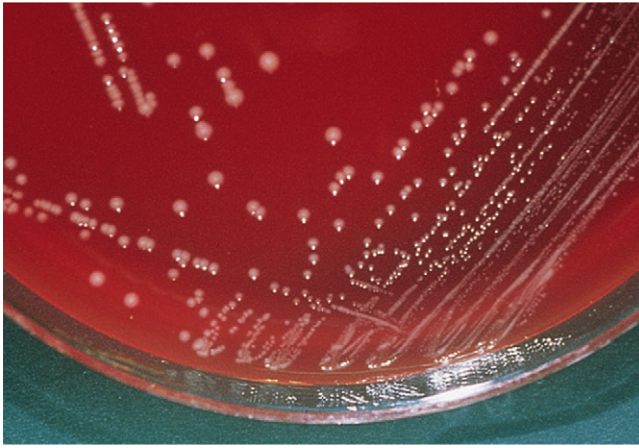


Fig. 16.3 *Corynebacterium diphtheriae* growing on sheep blood agar. (Courtesy Cathy Bissonette.)

Identification

The biochemical identification of medically important corynebacteria is outlined in Table 16.1. All medically important corynebacteria are catalase positive and nonmotile. CTBA is useful for differentiating corynebacteria because only *C. diphtheriae*, *C. belfantii*, *C. ulcerans*, and *C. pseudotuberculosis* form a brown halo as a result of cystinase activity. *C. diphtheriae* and *C. belfantii* are distinguished from the other two species by its lack of urease production, and *C. belfantii* can be differentiated from *C. diphtheriae* based on the ability to reduce nitrate. *C. diphtheriae* ferments glucose and maltose, producing acid, but not gas, and reduces nitrate to nitrite. The three biotypes of *C. diphtheriae*, *gravis*, *intermedius*, and *mitis*, can be separated using biochemical assays. A schematic diagram for presumptive identification of *C. diphtheriae* is shown in Fig. 16.4. MALDI-TOF mass spectrometry has been shown to accurately identify the potentially toxigenic strains of *Corynebacterium* spp., but it may not be able to differentiate *C. belfantii* from *C. diphtheriae*.

Test for toxigenicity

The identification of an isolate as *C. diphtheriae* does not mean that the patient is infected with a toxigenic strain. Determination of the presence of toxin is important for patient management and infection prevention. The growth medium and conditions markedly affect toxin production. The iron content of the medium must be growth rate limiting for full toxin production. The addition of iron to iron-starved cultures quickly inhibits toxin production. In vivo toxin testing is rarely done because the in vitro methods are reliable, less expensive, and free from animal use.

The in vitro diphtheria toxin detection procedure is an immunodiffusion test first described by Elek. In the Elek test, organisms (controls and unknowns) are streaked on medium of low iron content. Each organism is streaked in a single straight line parallel to each other and 10mm apart. A filter paper strip impregnated with diphtheria antitoxin is laid along the center of the plate on a line at right angles to the inoculum lines of control and unknown organisms (Fig. 16.5). The plate is incubated at 35° C and examined after 18, 24, and 48 hours. Lines of precipitation are best seen by transmitted light against a dark background. The white precipitin lines start about 4 to

5mm from the filter paper strip and are at an angle of about 45 degrees to the line of growth. If an isolate is positive for toxin production and it is placed next to the positive control, the toxin line of the positive control should join the toxin line of the positive unknown to form an arch of identity.

The Elek test requires that reagents and antisera be carefully controlled and titrated. Because of the difficulty of the test, it is performed only in certain reference laboratories. Rapid enzyme-linked immunosorbent assays and immuno-chromatographic strip assays are also available for the detection of diphtheria toxin. In addition, procedures for detecting the *C. diphtheriae* *tox* gene by polymerase chain reaction (PCR) have been developed by individual laboratories. The PCR assays can also be applied directly to clinical specimens.

Treatment

Diphtheria is treated by prompt administration of antitoxin. Commercial diphtheria antitoxin is produced from horse serum. Approximately 10% of patients who receive the antitoxin develop an allergic reaction to the horse serum. Consequently, hypersensitivity to horse serum precludes its administration. Antimicrobial agents have no effect on the toxin that is already circulating, but they do eliminate the focus of infection and prevent the spread of the organism. The drug of choice is penicillin; erythromycin is used for penicillin-sensitive individuals. Most patients do not develop immunity after infection; therefore vaccination should be administered after recovery.

Other corynebacteria

Corynebacterium amycolatum

C. amycolatum is one of the *Corynebacterium* species most frequently recovered from human specimens. It is part of the normal skin microbiota and has often been misidentified by clinical laboratories as *C. striatum*, *C. xerosis*, and *C. minutissimum*. It is often associated with prosthetic joint- or hardware-associated infections, and it has also been reported to cause mastitis, peritonitis, and bloodstream infection and endocarditis, typically in immunocompromised patients or in patients in a health care setting.

Colonies of *C. amycolatum* are flat and dry, have a matte or waxy appearance, and are nonlipophilic. Most strains have been reported to be resistant to a wide range of antimicrobials, including β -lactams, fluoroquinolones, macrolides, clindamycin, and aminoglycosides.

Corynebacterium jeikeium

C. jeikeium, named after Johnson and Kaye, who first linked this organism with human infections, appears to be part of the normal skin microbiota. Infections are typically limited to patients who are immunocompromised, have undergone invasive procedures, have central line catheters, or have prosthetic or other hardware devices. It is the most common cause of *Corynebacterium*-associated prosthetic valve endocarditis in adults. Infection is more likely to require valve replacement, and mortality rates as high as 33% have been reported in these patients. *C. jeikeium* also causes septicemia, meningitis, prosthetic joint infections, and skin complications, such as rash and subcutaneous nodules.

Table 16.1 Identification of corynebacteria

Characteristic	<i>C. diphtheriae</i>	<i>C. belfantii</i>	<i>C. amycolatum</i>	<i>C. jeikeium</i>	<i>C. pseudodiphtheriticum</i>	<i>C. pseudotuberculosis</i>	<i>C. striatum</i>	<i>C. ulcerans</i>	<i>C. urealyticum</i>
Tinsdale halo	+	+	–	–	–	+	–	+	–
Lipophilic	V	V	–	+	–	–	–	–	+
β -Hemolysis	V	V	–	–	–	+	–	V	–
CAMP reaction	–	–	–	–	–	Reverse	V	Reverse	–
Nitrate reduction	+	–	V	–	+	V	+	–	–
Urease	–	–	V	–	+	+	–	+	+
Alkaline phosphatase	+	+	+	V	V	V	+	+	V
Gelatin hydrolysis	–	–	–	–	–	–	–	+	–
Fermentation/oxidation	F	F	F	O	O	F	F	F	O
Acid production from:									
Glucose	+	+	+	+	–	+	+	+	–
Maltose	+	+	V	V	–	+	–	+	–
Sucrose	–	–	V	–	–	V	V	–	–

+, Present or positive; –, absent or negative; F, Fermentative; O, oxidative; V, variable.

CAMP, Christie, Atkins, Munch-Peterson.

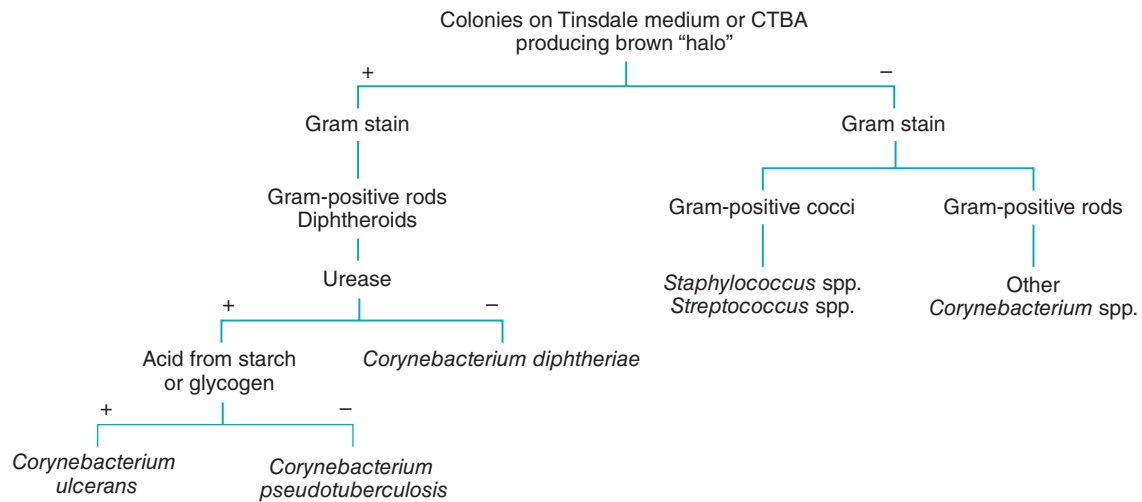


Fig. 16.4 Schematic diagram for the presumptive identification of *Corynebacterium diphtheriae*. CTBA, Cystine-tellurite blood agar.

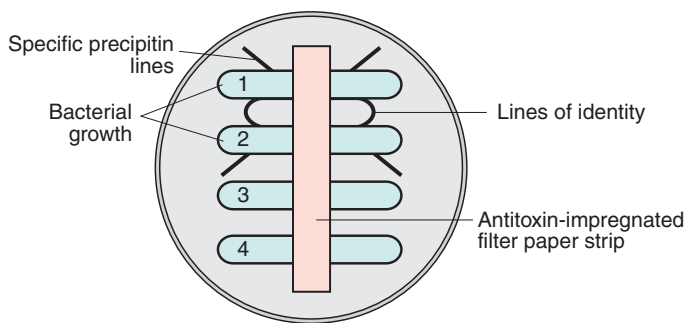


Fig. 16.5 Elek test for toxin-producing strains of *Corynebacterium diphtheriae*. 1, Positive control; 2, unknown (toxigenic); 3, negative control; 4, unknown (nontoxigenic).

The organism is lipophilic and a strict aerobe that is non-hemolytic, does not produce urease, and is nitrate reduction negative. *C. jeikeium* has been reported to be resistant to a wide range of antimicrobials, including penicillins, cephalosporins, macrolides, and aminoglycosides. Multidrug resistance cannot be used as the only identifying characteristic because several *Corynebacterium* species, such as *C. amycolatum*, exhibit similar multidrug-resistance patterns. The drug of choice for treating *C. jeikeium* infections is vancomycin.

Corynebacterium pseudodiphtheriticum

C. pseudodiphtheriticum, which is part of the normal biota of the human nasopharynx, is an infrequent cause of infection. It is most frequently associated with respiratory tract infections in immunocompromised individuals or patients with other underlying diseases, such as chronic obstructive pulmonary disease or diabetes mellitus. It was identified in association with ventilator-associated pneumonia in cases of coronavirus disease 2019 (COVID-19). In some cases, respiratory tract infection can mimic respiratory diphtheria. In addition, *C. pseudodiphtheriticum* has been reported to cause endocarditis, urinary tract infections (UTIs), and cutaneous wound infections in immunocompromised patients. In contrast with other corynebacteria, *C. pseudodiphtheriticum* does not show the characteristic pleomorphic morphology. The cells stain evenly and often appear in palisades. The species grows well on standard laboratory media, reduces nitrate, and produces urease.

Corynebacterium pseudotuberculosis

C. pseudotuberculosis is primarily a veterinary pathogen. Human infections typically have been associated with contact with sheep and are rare. *C. pseudotuberculosis* causes a granulomatous lymphadenitis in humans. The organism produces a dermonecrotic toxin that causes death of various cell types, and it can produce diphtheria toxin. Similarly to *C. diphtheriae*, *C. pseudotuberculosis* also produces a brown halo on CTBA. *C. pseudotuberculosis* produces urease and on SBA forms small, yellowish-white colonies.

Corynebacterium striatum

C. striatum is part of the human skin and the nasopharynx microbiota and is frequently isolated in clinical microbiology laboratories. This organism is often considered a commensal organism or skin "contaminant." However, hospital-acquired infections have been reported, and clinical case reports support the characterization of this organism as an emerging pathogen in immunocompromised as well as immunocompetent patients. *C. striatum* has been most commonly associated with device-related infection and has been reported in cases of endocarditis, septic arthritis, meningitis, and pneumonia. The organism is nonlipophilic and pleomorphic, and it often produces small, shiny, convex colonies in about 24 hours, which grow to appear striated with continued incubation. *C. striatum* has shown resistance to penicillins and other β -lactams, macrolides, and fluoroquinolones but is typically susceptible to vancomycin. Resistance to daptomycin, including high-level resistance (MIC = >256 μ g/mL), has been reported recently.

Corynebacterium ulcerans

Although an infrequent cause of infection, *C. ulcerans* has been isolated from humans with diphtheria-like illness, and a significant number of isolates produce diphtheria toxin. It has been isolated from skin ulcers and exudative pharyngitis. This organism is also a veterinary pathogen, causing mastitis in cattle and other domestic and wild animals. Human infections are usually acquired through contact with animals or by ingestion of unpasteurized dairy products. It produces a brown halo around colonies on CTBA. The organisms grow well on SBA and produce a narrow zone of β -hemolysis.

C. ulcerans is urease positive and does not reduce nitrate, differentiating it from *C. diphtheriae*.

Corynebacterium urealyticum

C. urealyticum is most commonly associated with UTIs. This organism is lipophilic and is a strict aerobe. Presumptive identification of *C. urealyticum* can be made for urine isolates with pinpoint, nonhemolytic, white colonies that have characteristic coryneform microscopic morphology. *C. urealyticum* is nitrate negative, catalase positive, and urease positive within minutes after inoculation on a Christensen urea slant. Resistance has been reported for a broad range of antimicrobials, including β -lactams, aminoglycosides, and trimethoprim-sulfamethoxazole. Some isolates have also demonstrated resistance to fluoroquinolones, macrolides, and tetracycline. The drug of choice for *C. urealyticum* infections is vancomycin.

Identification of coryneform bacteria

Coryneform bacteria are often identified to the species level (1) if they are isolated from normally sterile sites, particularly cerebrospinal fluid and from two or more blood cultures; (2) if they are the predominant organism from properly collected (not contaminated with commensal microbiota) clinical material; (3) if they are the predominant organism from urine samples when the total colony count is greater than 10^5 /mL or if they are the sole isolate with a colony count greater than 10^4 /mL; and (4) for *Corynebacterium* spp. if they are the only isolate in patients with symptomatic pneumonia. Because the clinical significance of *Corynebacterium* spp. can differ, details regarding the specimen type, method of collection, and other patient-related factors should be taken into consideration for identification of these organisms.

In addition to conventional biochemical testing, commercial identification systems presently available include the RapID CB Plus system (Thermo Scientific, Waltham, MA), API Rapid Coryne system (bioMérieux, Durham, NC), Microscan ID panel (Beckman Coulter, Sacramento, CA), BBL Crystal GP system (BD Diagnostics, Sparks, MD), and VITEK 2 ANC card (bioMérieux). Although these systems have short incubation times ranging from a few hours to 48 hours, users should keep the limitations of these systems in mind. The test systems perform best with species that grow rapidly in ambient air and do not require nutritional supplements. Accurately identifying less frequently isolated species is difficult with many of these systems.

Many clinical microbiology laboratories, especially in academic medical centers and reference laboratories, use MALDI-TOF mass spectrometry for the identification of bacteria isolated from clinical cultures (see Chapter 11). This technology is rapid and has the ability to accurately identify many *Corynebacterium* isolates to the species level compared with biochemical systems. Studies have reported 89% to 94% accuracy in identification to the species level. However, accuracy of identification is organism and system dependent; therefore some species with similar deoxyribonucleic acid (DNA) sequences are not clearly differentiated by MALDI-TOF mass spectrometry. It is recommended that clinically significant, unidentifiable coryneform bacteria be sent to a reference laboratory for complete characterization. Many reference laboratories now use MALDI-TOF mass spectrometry or 16S DNA gene sequencing to identify species of coryneform bacteria and other coryneform bacteria.

Rothia

Rothia spp., which are actually gram-positive cocci that can appear rodlike, belong to the family Micrococcaceae. Of the greater than 10 species in the genus, at least 3 are regarded as clinically significant. *Rothia mucilaginosa* has been linked to bacteremia, endocarditis, pneumonia, and other infections. *R. mucilaginosa* is usually coccoid on Gram stain. Colonies of *R. mucilaginosa* can be whitish or gray, mucoid, and are often “sticky” (adherent to the agar). *Rothia dentocariosa*, which is typically, but not always, rod shaped, is a member of the normal human oropharyngeal microbiota and may be found in saliva and supragingival plaque. It has been isolated from patients with endocarditis. Microscopically, this organism resembles coryneform bacilli, forming not only short, gram-positive bacilli but also branching filaments that resemble filaments of facultative actinomycetes. However, when placed in broth, the species produces coccoid cells, a characteristic differentiating it from actinomycetes. Colonies of *R. dentocariosa* are usually white and may be either smooth or rough. Over time, the colonies may look wrinkled. *Rothia aeria* was first described in 2004 after the organism was isolated from the air in the Russian space station Mir. Since then, *R. aeria* has been recovered from abscesses from various sites, bacteremia, endocarditis, joint infections, and respiratory infections. Microscopically *R. aeria* may also form rods or filaments. Colonies of *R. aeria* are initially white and smooth but become dry and wrinkled. The colonies may adhere to the agar. *R. mucilaginosa*, *R. dentocariosa*, and *R. aeria* are members of the human oral biota. Other members of the genus are environmental. *Rothia* is nitrate positive, nonmotile, esculin hydrolysis positive, and urease negative. The catalase reaction is variable for most *Rothia* strains.

Related genera

Numerous related genera are occasional isolates in clinical laboratories; many are thought to belong to the normal biota and are generally considered commensal organisms or are environmental. Examples include *Brevibacterium*, *Cellulomonas*, *Microbacterium*, *Janibacter*, *Oerskovia*, *Paraoerskovia*, and *Dermabacter*.

Listeria monocytogenes

General characteristics

The genus *Listeria* contains over 20 species. Only *Listeria monocytogenes* is considered an important human pathogen, whereas *Listeria ivanovii* is primarily an animal pathogen but has been linked to rare systemic infections in humans with underlying conditions. *L. monocytogenes* is widespread in the environment and has been recovered from soil, water, vegetation, and animal products, such as raw milk, cheese, poultry, and processed meats. It also has been isolated from crustaceans, flies, and ticks. *L. monocytogenes* has long been known to cause illness in many species of wild and domestic animals, including sheep, cattle, swine, horses, dogs, cats, rodents, birds, and fishes, and it can be isolated from both human and animal asymptomatic carriers.

Listeriosis is recognized as an uncommon but serious infection primarily of neonates, pregnant women, older adults, and immunocompromised hosts. Infection may also occur in

healthy individuals. Each year, approximately 1600 cases of listeriosis are reported in the United States, resulting in 260 deaths. Large, multistate outbreaks of illness caused by *L. monocytogenes* have occurred several times, usually associated with foods, such as various types of cheese or deli meats. One of the larger multistate outbreaks, which was linked to cantaloupes from a Colorado farm, occurred in the United States in 2011 and resulted in 147 cases and 33 deaths.

Virulence factors

L. monocytogenes produces many products that have been proposed as virulence factors. These include internalin, listeriolysin O, phospholipase C, actin polymerizing protein, superoxide dismutase, and a surface protein (p60). Once ingested, *L. monocytogenes* survives initial host defenses such as proteolytic enzymes and low pH. The organism then adheres to and invades host cells with proteins from the internalin family. When invading a host cell, *L. monocytogenes* becomes trapped in a vacuole. The organism then produces listeriolysin O, a hemolysin, which forms a pore in the vacuole and facilitates escape into the cytoplasm. The correlation between listeriolysin O production and virulence is strong; strains without listeriolysin O are avirulent. Phospholipase C is also involved in *L. monocytogenes* escape from the vacuole. When the organism reaches the cytoplasm, it infects other cells with the aid of actin polymerizing protein. This protein polymerizes actin molecules into actin filaments. *L. monocytogenes* uses these filaments to move to adjacent cells. The protein p60 induces phagocytosis through increased adhesion and penetration into mammalian cells.

Clinical infections

The infectious dose of listeriosis is not well understood. The majority of infections are caused by ingestion of contaminated food products such as meat, poultry, cheese, and raw milk with subsequent spread through the intestine. *L. monocytogenes* appears to have a tropism for the central nervous system (CNS), and a high mortality rate (20% to 50%) is seen in patients with CNS infections. The clinical manifestations of listeriosis differ among patient groups. Infections of newborns and immunocompromised adults are the most common, but disease in healthy individuals, particularly in pregnant women, also occurs.

Disease in pregnant women

During pregnancy, listeriosis is most commonly seen during the third trimester. It has been postulated that *L. monocytogenes* is responsible for spontaneous abortion and stillborn neonates. A pregnant woman with listeriosis may experience a flulike illness with fever, headache, and myalgia. At this point, the organism is in the bloodstream and has seeded the uterus and the fetus. It may progress and result in premature labor or septic abortion within 3 to 7 days. It appears that the infection often is self-limiting because the source of the infection is eliminated when birth occurs.

Disease in the newborn

Infection of neonates with *L. monocytogenes* is extremely serious; fatality rates approach 50% if the fetus is born alive. Similar to *Streptococcus agalactiae* neonatal disease, there are two forms of neonatal listeriosis: early onset and late onset. Early-onset listeriosis results from an intrauterine infection that can cause illness at or shortly after birth. The result is most often sepsis. Early-onset disease may be associated with aspiration of infected amniotic

fluid. Late-onset disease occurs several days to weeks after birth. Affected infants generally are full-term infants and healthy at birth. The disease is most likely to manifest as meningitis. The fatality rate is lower than in early-onset infection, although it also is a very serious, potentially fatal infection.

Disease in the immunosuppressed host

Invasive listeriosis most commonly occurs in persons who are immunosuppressed or in older adults and particularly in patients receiving chemotherapy. Young children are also at risk for infection. In older adults and immunocompromised persons, the fatality rate is high. The most common manifestations are CNS infection and endocarditis. Diagnosis is made by culturing *L. monocytogenes* from blood or CSF.

Infection of apparently healthy individuals may occur through the intestinal tract when they eat food contaminated with *L. monocytogenes*. Outbreaks have occurred as a result of eating contaminated cheese, coleslaw, and chicken. Contaminated ice cream, hot dogs, and luncheon meats have served as vehicles for this foodborne disease. The penicillins, aminoglycosides, and macrolides have been effectively used to treat listeriosis. For invasive disease, ampicillin or penicillin in combination with gentamicin is the preferred treatment. Cephalosporins are inactive against *L. monocytogenes*. Resistance is uncommon, although some strains are resistant to one or more agents.

Case check 16.1

The Case in Point describing invasive disease in an older, immunosuppressed patient is typical for *L. monocytogenes* infection. Also, the phenotypic characteristics of the isolate are most consistent with *L. monocytogenes*. *L. monocytogenes* is β -hemolytic on SBA, catalase positive, motile at room temperature but not at 35°C, esculin hydrolysis positive, hippurate hydrolysis positive, and block shape positive with the CAMP test. Typically, most people who contract *L. monocytogenes* infection consume contaminated food, such as dairy products or meat.

Laboratory diagnosis

Microscopy

In direct smears (Fig. 16.6), *L. monocytogenes* appears as a gram-positive coccobacillus. With subculturing, cells become coccoidal (Fig. 16.7). Older cultures often appear gram variable. Cells may be found singly, in short chains, or in palisades. Depending on the culture conditions, *L. monocytogenes* can resemble *Streptococcus* when found in the coccoid form and *Corynebacterium* when the bacillus forms prevail. Organisms are not usually seen on the CSF smear.

Cultural characteristics

L. monocytogenes grows well on SBA and chocolate agar as well as on nutrient agars and in broths, such as brain-heart infusion medium and thioglycolate broth. The organism prefers a slightly increased carbon dioxide (CO₂) tension for isolation. The colonies are small, round, smooth, and translucent. They are surrounded by a narrow zone of β -hemolysis, which may be visualized only if the colony is removed. The colonies and hemolysis resemble those seen with *S. agalactiae* (Fig. 16.8A).

The optimal growth temperature for *L. monocytogenes* is 30°C to 35°C, but growth occurs over a wide range (0.5°C to 45°C).

Because *L. monocytogenes* grows at 4° C, a technique called **cold enrichment** may be used to isolate the organism from polymicrobial clinical specimens. The specimen is placed into broth and incubated at 4° C for several weeks. Subcultures are made at weekly intervals and examined for *L. monocytogenes*. Most bacteria grow more slowly than *L. monocytogenes* at this

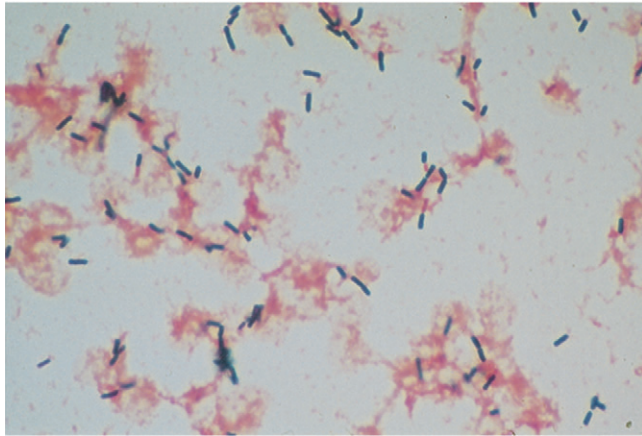


Fig. 16.6 Gram stain of *Listeria monocytogenes* in the blood (×1000). (Courtesy Cathy Bissonette.)

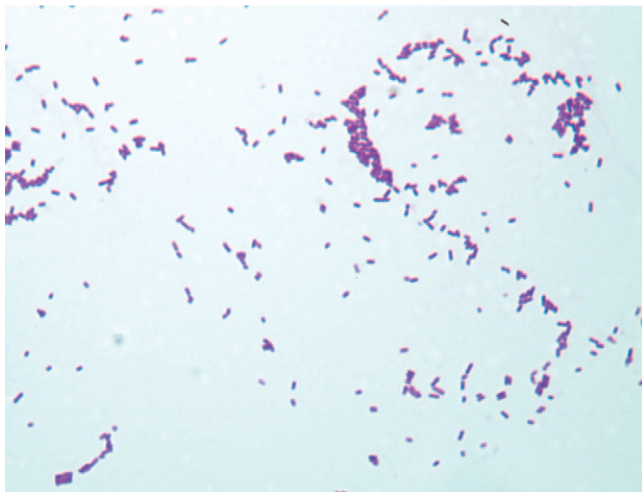


Fig. 16.7 Gram stain of *Listeria monocytogenes* from a culture (×1000). (Courtesy Steve Mahlen and Amanda Harrington.)

temperature. The length of time required for isolation using this method lessens its importance in the clinical setting because treatment must begin early in the infectious process.

Identification

The diagnosis of listeriosis depends on isolating *L. monocytogenes* from **blood, CSF, or swabs taken from lesions**. Table 16.2 lists the characteristics of *L. monocytogenes* and bacteria with similar colony morphologies. *L. monocytogenes* is **hippurate hydrolysis positive like *S. agalactiae*, but unlike *S. agalactiae*, it is catalase positive, bile esculin hydrolysis positive, and motile at room temperature**. In wet mount preparations, *L. monocytogenes* exhibits **tumbling motility (end-over-end motility) when viewed microscopically**. In motility medium, the characteristic **“umbrella” pattern** is seen when the organism is incubated at room temperature (22° to 25° C) but not at 35° C (Fig. 16.9).

L. monocytogenes produces a positive CAMP reaction; a more pronounced CAMP reaction is seen with *L. monocytogenes* when *Rhodococcus equi* is used in place of *Staphylococcus aureus*. *L. monocytogenes* produces a **“block”-type hemolysis with the CAMP test**. This type of hemolysis is in contrast with the **“arrowhead” type produced by *S. agalactiae* (group B *Streptococcus*)** (see Fig. 16.8B). The positive CAMP reaction distinguishes *L. monocytogenes* from other *Listeria* spp., which are CAMP test negative. A presumptive identification can be made on the basis of the results of the Gram stain, tumbling motility, positive catalase, and esculin hydrolysis. Confirmatory findings include acid production from glucose and positive Voges-Proskauer and methyl red reactions. *L. monocytogenes* and other *Listeria* species are identified well by MALDI-TOF mass spectrometry.

Non-spore-forming, nonbranching, catalase-negative bacilli

Erysipelothrix rhusiopathiae

General characteristics

There are six species in the genus *Erysipelothrix*: *Erysipelothrix rhusiopathiae*, *E. tonsillarum*, *E. inopinata*, *E. larvae*, *E. muris*, and *E. piscisicarius*. *E. rhusiopathiae* is the only species in the genus known to cause disease in humans. **It is a gram-positive, catalase-negative, non-spore-forming, pleomorphic rod that has a tendency to form long filaments**. It is found worldwide and

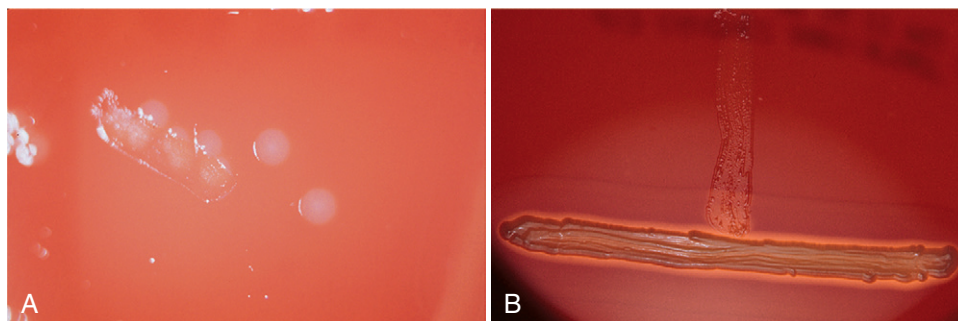


Fig. 16.8 **A**, β -Hemolysis: *Listeria* on sheep blood agar (SBA) plate. *Listeria monocytogenes* growing on SBA with colony morphology similar to that of group B β -hemolytic streptococci. **B**, Conventional Christie, Atkins, Munch-Peterson (CAMP) test with *L. monocytogenes* showing “block” hemolysis (enhanced hemolysis in the shape of a block) at the junction with the *Staphylococcus aureus* inoculum.

Table 16.2 Differentiation of *Listeria monocytogenes* and other gram-positive bacteria

Organism	Catalase	Esculin hydrolysis	Motility	β -hemolysis	Growth in 6.5% NaCl
<i>Listeria monocytogenes</i>	+	+	+	+	+
<i>Corynebacterium</i> spp.	+	—	V	V	V
<i>Streptococcus agalactiae</i>	—	—	—	+	V
<i>Enterococcus</i> spp.	— ^a	+	—	V	+

+, Present or positive; —, absent or negative; V, variable.

^aMay be weak.

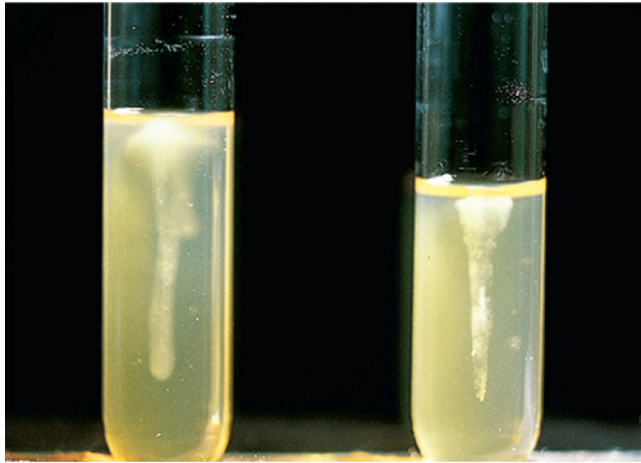


Fig. 16.9 Umbrella motility: *Listeria*. Motility test for *Listeria monocytogenes* showing the typical “umbrella” pattern that occurs toward the surface of the medium when this organism is incubated at room temperature. The tube on the *left* is positive; the tube on the *right* is a negative control.

is a commensal or a pathogen in a wide variety of vertebrates and invertebrates, including domestic swine, birds, and fishes. Human cases typically result from occupational exposure. Individuals whose work involves handling fish and animal products are most at risk. The usual route of infection is through cuts or scratches on skin. The organism is resistant to salting, pickling, and smoking, and it survives well in environmental sources such as water, soil, and plant material.

Clinical infections

E. rhusiopathiae produces three types of disease in humans: erysipelas, which is a localized skin disease; septicemia, which is often associated with endocarditis; and a generalized, diffuse cutaneous infection. The incidence of *E. rhusiopathiae* infections is low. Systemic infection is uncommon and rarely develops from erysipelas. Endocarditis has been seen in patients who have had valve replacements as well as in individuals with apparently normal heart valves. Risk factors for endocarditis include a history of heart disease and a history of alcohol abuse. Endocarditis caused by *E. rhusiopathiae* has a 38% mortality rate, and approximately one third of patients who develop endocarditis must undergo valve replacement.

Erysipelas, the most common infection caused by *E. rhusiopathiae* in humans, is a localized skin infection that resembles streptococcal erysipelas. The lesions usually are seen on the hands or fingers because the organisms usually are inoculated through work activities. The incubation period is 2 to 7 days.

The infected area is painful and swollen and gives rise to a characteristic lesion—a sharply defined, slightly elevated, purplish red zone that spreads peripherally as discoloration of the central area fades. Low-grade fever, arthralgia, lymphangitis, and lymphadenopathy may occur. Erysipelas is a self-limiting infection that normally heals within 3 to 4 weeks but may continue for months. Relapses are known to occur.

The generalized, diffuse cutaneous disease caused by *E. rhusiopathiae* is rare and manifests itself as an exacerbation of the erysipelas lesion. This cutaneous disease tends to last longer than erysipelas and relapses as well. Penicillin or amoxicillin are the drugs of choice for treating both cutaneous and systemic infections. *E. rhusiopathiae* is routinely resistant to vancomycin.

Laboratory diagnosis

Microscopy

E. rhusiopathiae is a thin, rod-shaped, gram-positive organism that can form long filaments (Fig. 16.10). It is arranged singly, in short chains, or in a V shape. The last arrangement is similar to that seen with corynebacteria. *E. rhusiopathiae* decolorizes easily, so it may appear gram variable.

Culture characteristics

Typical specimens received for the isolation of *E. rhusiopathiae* include tissue biopsy or aspirates from skin lesions. These should be inoculated into a nutrient broth with 1% glucose and incubated in 5% CO₂ at 35° C. The organism also grows on standard culture media, including SBA and chocolate agar. Subcultures from both should be inoculated daily onto SBA plates. On SBA, the colonies are usually nonhemolytic and pinpoint after 24 hours of incubation. The colonies of *E. rhusiopathiae* and *L. monocytogenes* are compared in Fig. 16.11. After 48 hours of incubation, two distinct colony types are seen: A smaller, smooth form is transparent, glistening, and convex with entire edges; the larger, rough colonies are flatter with a matte surface, curled structure, and irregular edges. The colonies often appear α -hemolytic after a few days of growth. In addition, *E. rhusiopathiae* grows in blood culture media from systemic infections.

Identification

Table 16.3 lists the characteristics of *Erysipelothrix* and similar gram-positive bacilli. A catalase-negative, nonmotile, pleomorphic, aerobic or facultatively anaerobic, gram-positive rod that is hydrogen sulfide positive is suggestive of *E. rhusiopathiae*. In addition, the organism is Voges-Proskauer test negative. Growth of *E. rhusiopathiae* in a gelatin stab culture yields a highly characteristic “test tube brush-like” pattern at 22° C. *E. rhusiopathiae* is identified well by MALDI-TOF mass spectrometry.

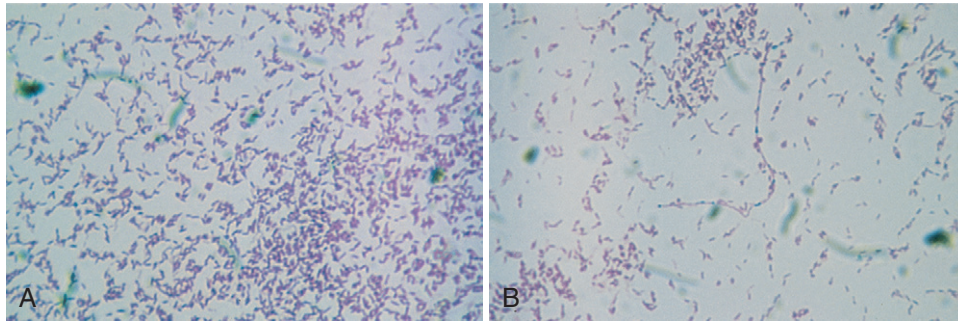


Fig. 16.10 A, Gram-stained smear of *Erysipelothrix rhusiopathiae* at 24 hours ($\times 1000$). B, Gram-stained smear of *E. rhusiopathiae* at 72 hours showing the tendency to form long filaments, which are easily decolorized ($\times 1000$). (Courtesy Cathy Bissonette.)

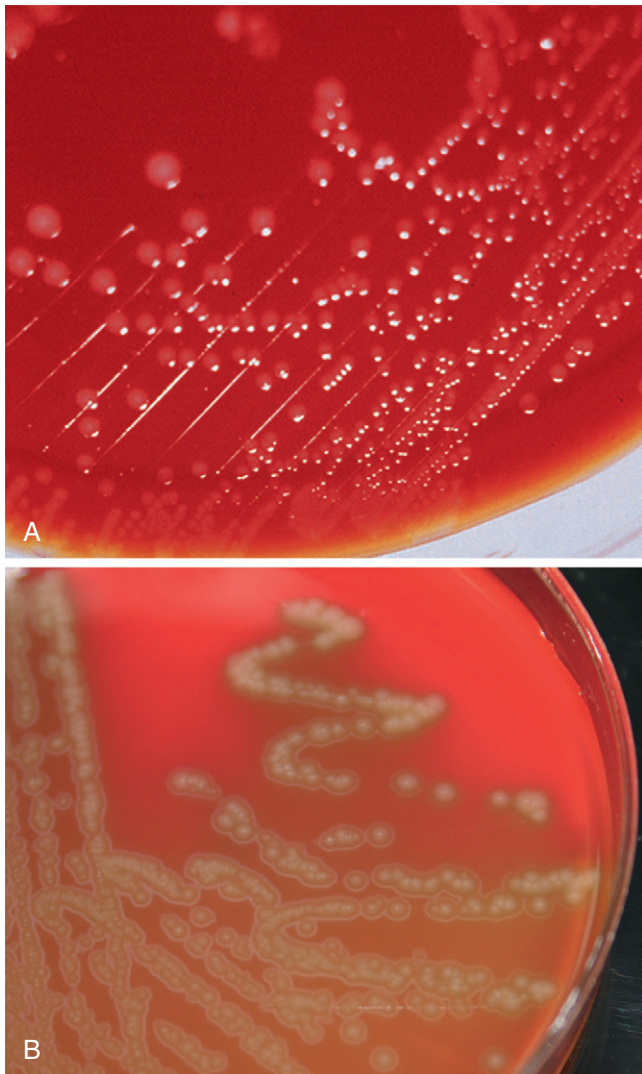


Fig. 16.11 Comparison of colony morphology of *Listeria* (A) and *Erysipelothrix* (B) growing on sheep blood agar after 24 hours of incubation. (Courtesy Cathy Bissonette.)

Arcanobacterium and Trueperella

In 2011, some members of the genus *Arcanobacterium* were moved to the genus *Trueperella*. The primary human pathogen of the genus *Arcanobacterium* is *A. haemolyticum*. *Trueperella pyogenes* and *Trueperella bernardiae* can also cause infections in

humans. *T. pyogenes* is an animal pathogen that is best known for causing infections in cattle. In humans, *T. pyogenes* is a rare cause of infections, such as sepsis and wound infections, and infections typically occur in individuals who have had contact with animals. *T. bernardiae*, also a rare cause of infections in humans, has been associated with bacteremia, wound infections, UTIs, and septic arthritis.

A. haemolyticum has been recovered from patients 10 to 20 years of age with pharyngitis and must be distinguished from *C. diphtheriae* and *C. ulcerans* as well as group A streptococci. Pharyngitis caused by *A. haemolyticum* can be mild or severe and is often clinically indistinguishable from pharyngitis caused by β -hemolytic streptococci. Most patients develop cervical lymphadenopathy, and approximately 50% of patients develop a pruritic, scarlatiniform rash and desquamation of the skin of the hands and feet. In addition, *A. haemolyticum* has been associated with soft tissue infections, sepsis, endocarditis, and other infections.

A. haemolyticum and clinically significant *Trueperella* spp. are catalase negative. *A. haemolyticum* produces small colonies on SBA that demonstrate a narrow zone of β -hemolysis after 24 to 48 hours of incubation similar in appearance to β -hemolytic streptococci (Fig. 16.12). Frequently, a black opaque dot is observed on the agar when the colony is scraped away. Pitting of the agar beneath the colony has also been reported. Gram staining of the isolated colony quickly rules out group A streptococci. Many of the rods are pleomorphic, and some cells may demonstrate rudimentary branching (Fig. 16.13). *A. haemolyticum* is both lipase and lecithinase positive. It exhibits a reverse CAMP reaction (CAMP inhibition reaction) because the hemolysis produced by a β -lysin-producing *S. aureus* is inhibited by a phospholipase D excreted by *A. haemolyticum*. They are CAMP test positive if *S. agalactiae* is used in place of *S. aureus*. All of these bacteria are identified well by MALDI-TOF mass spectrometry. Azithromycin is the drug of choice for treatment.

Gardnerella vaginalis

General characteristics

The genus *Gardnerella* includes four species. *G. vaginalis*, first described in 1953, is the primary species in the genus associated with human infections. It is found as normal biota in the human urogenital tract. *G. vaginalis* is a short, pleomorphic gram-positive rod or coccobacillus that often stains gram variable or gram negative. *G. vaginalis* has a gram-positive type of cell wall; however, the peptidoglycan layer is

Table 16.3 Characteristics of *Listeria*, *Corynebacterium*, *Erysipelothrix*, and similar gram-positive bacilli

Organisms	Catalase production	Motility	Esculin hydrolysis	Acid from glucose	H ₂ S from TSI agar	Hemolysis type	Nitrate reduction	Urease production
<i>Corynebacterium</i> spp.	+	–	V	V	–	V	V	V
<i>Listeria monocytogenes</i>	+	+ ^a	+	+	–	β	–	–
<i>Erysipelothrix rhusiopathiae</i>	–	–	–	+	+	None, α	–	–
<i>Arcanobacterium haemolyticum</i>	–	–	–	+	–	β	–	–
<i>Gardnerella vaginalis</i>	–	–	–	+	–	None ^b	V	+
<i>Rhodococcus</i> spp.	+	–	–	–	–	None	V	+
<i>Rothia dentocariosa</i>	+	–	+	+	–	None	+	–

+, Present or positive; –, absent or negative; H₂S, hydrogen sulfide; TSI, triple sugar iron; V, variable.

^aMotile at 25° C.

^bβ-Hemolytic on human blood agar but nonhemolytic on sheep blood agar.

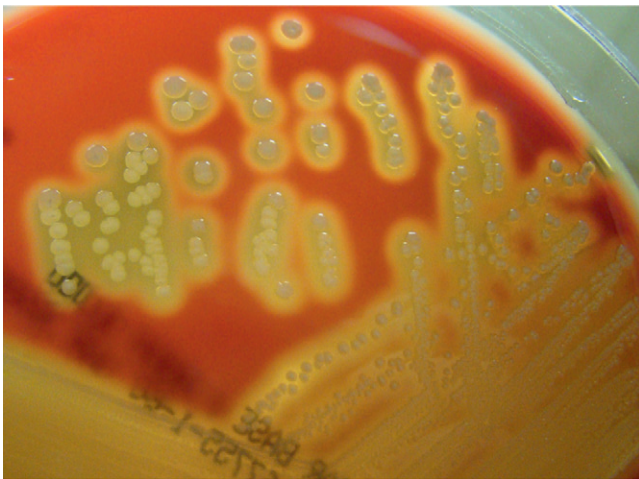


Fig. 16.12 *Arcanobacterium haemolyticum* growing on sheep blood agar. (Courtesy Steve Mahlen and Amanda Harrington.)

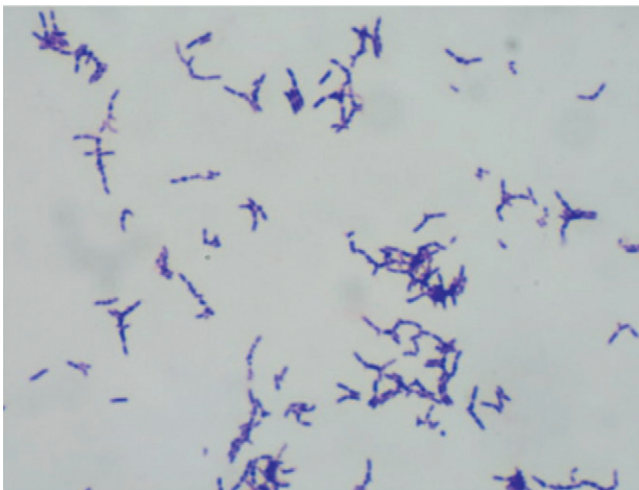


Fig. 16.13 Gram stained smear of *Arcanobacterium haemolyticum* from a colony (×1000). (Courtesy Steve Mahlen and Amanda Harrington.)

thinner than that found in other gram-positive bacteria such as *Corynebacterium* and *Lactobacillus*.

Clinical infections

G. vaginalis is primarily known for its association with **bacterial vaginosis** (BV) in humans, the most common vaginal condition in women 15 to 44 years of age. For years, *G. vaginalis* was thought to be the only cause of BV. However, the organism can be isolated from 40% of women without BV. More than likely, BV is a polymicrobial disease in which *G. vaginalis* and other bacteria, such as *Prevotella* spp., *Peptostreptococcus* spp., *Bacteroides* spp., *Porphyromonas* spp., *Mobiluncus* spp., *Megasphaera* spp., *Sneathia* spp., *Fusobacterium* spp., *Atopobium vaginae*, *Mycoplasma hominis*, *Ureaplasma urealyticum*, and members of the class *Clostridia*, are involved. BV is characterized by a malodorous discharge and vaginal pH greater than 4.5. BV generally results from a reduction in the *Lactobacillus* population in the vagina, followed by an increase in vaginal pH; this results in overgrowth of BV-associated organisms that make up the normal vaginal biota. *G. vaginalis* can also play a role in UTIs in men and women. The organism has rarely been isolated from other clinical sources, such as blood cultures or wounds. The drug of choice to treat BV is metronidazole, although clindamycin is also often used.

Laboratory diagnosis

Microscopy

Gram staining of vaginal secretions is generally regarded as the reference method for diagnosing BV. The observation of “clue cells,” large squamous epithelial cells with gram-positive and gram-variable bacilli and coccobacilli clustered on the edges, aids the diagnosis of BV, particularly if *Lactobacillus* rods are absent in the wet mount. *G. vaginalis* often appears as a pleomorphic, gram-variable coccobacillus or short rod. The cells often stain gram negative and are 1.5 to 2.5 μm in length. The Nugent scoring system for Gram-stained vaginal smears is a more accurate means of diagnosing BV than cultures (Table 16.4). Stained smears are examined and scored for the presence of *Lactobacillus*, *Gardnerella*, and *Mobiluncus* morphotypes. **In addition, clue cells can be visualized in wet mounts of vaginal fluid when BV is suspected.**

Additional identification methods

Amsel's clinical criteria can also be used to diagnosis BV if three of four criteria are found: (1) homogeneous, thin, white discharge that smoothly coats the vaginal walls, (2) clue cells, (3) pH of vaginal fluid greater than 4.5, and (4) fishy odor of vaginal discharge before or after addition of 10% potassium hydroxide—the whiff test. In addition, the Affirm VP III test (Becton Dickinson, Sparks, MD), a DNA hybridization probe test for *G. vaginalis*, and the OSOM BV Blue test (Sekisui Diagnostics, Framingham, MA), which detects vaginal fluid sialidase activity, have acceptable performance compared with Gram staining of vaginal material.

Culture characteristics

Because *G. vaginalis* can be found as normal vaginal biota and its role in BV is questionable, cultures for *G. vaginalis* are infrequently performed. Vaginal discharge collected from suspected BV cases is the most common specimen used for the isolation of *G. vaginalis*. Because it is part of the urogenital microbiota, the organism can also be isolated from urine. It often takes longer than 24 hours to develop visible colonies, and *G. vaginalis* grows best in 5% to 7% CO₂ at a temperature of 35° to 37° C. *G. vaginalis* grows on SBA as pinpoint, nonhemolytic colonies. It also grows on chocolate agar. Additional media for the recovery of *G. vaginalis* include human blood bilayer Tween (HBT) agar or *Gardnerella* selective V (*vaginalis*) agar that also contains human blood. When cultured on human blood, colonies are β-hemolytic, small, gray, and opaque. *G. vaginalis* also produces β-hemolytic colonies on media made with rabbit blood but not sheep blood.

Identification

See Table 16.3 for a list of the key biochemical characteristics of *G. vaginalis* and other gram-positive bacilli. β-Hemolytic colonies on HBT agar should be suspected as *G. vaginalis*. MALDI-TOF mass spectrometry will identify this organism well. Some laboratories use 16S rRNA gene sequencing for definitive identification of *G. vaginalis*.

Table 16.4 Nugent scoring system for Gram-stained vaginal smears

Lactobacillus morphotypes (boxy, gram-positive bacilli)		Gardnerella and Bacteroides morphotypes (pleomorphic, gram-variable and gram-negative, short bacilli)		Mobiluncus morphotypes (curved, gram-variable bacilli)	
Quantity	Points	Quantity	Points	Quantity	Points
4+	0	0	0	0	0
3+	1	1+	1		
2+	2	2+	2	1+ to 2+	1
1+	3	3+	3		
0	4	4+	4	3+ to 4+	2

Interpretation: total score 0 to 3, normal vaginal microbiota; 4 to 6, indeterminate for bacterial vaginosis; 7 to 10, bacterial vaginosis.

Modified from Nugent, R. P., et al. (1991). Reliability of diagnosing bacterial vaginosis is improved by a standardized method of Gram stain interpretation. *J Clin Microbiol*, 29, 297.

Non-spore-forming, branching, aerobic actinomycetes

Nocardia

General characteristics

Nocardia spp. are aerobic, branched, beaded, gram-positive bacilli. The beads are not usually spaced at consistent intervals (Fig. 16.14A). In many instances, finely beaded, branching rods are a primary clue that a clinical sample contains *Nocardia* spp. In addition, *Nocardia* spp. are partially acid fast, meaning they retain the primary stain but only when a weak acid is used as the decolorizer during the acid-fast staining process (see Fig. 16.14B). This characteristic is also referred to as modified acid-fast positive. The acid-fast stain is used to visualize the mycobacteria and is discussed in Chapter 26.

The colony and microscopic morphology as well as the types of infections caused sometimes resemble those of fungi, but these organisms are true bacteria. *Nocardia* spp. can be recovered on standard nonselective media; however, growth may take 1 week or longer. Most *Nocardia* spp. are found worldwide in soil and on plant material, and several have been implicated in human infections. Generally, infections caused by *Nocardia* occur in immunocompromised patients.

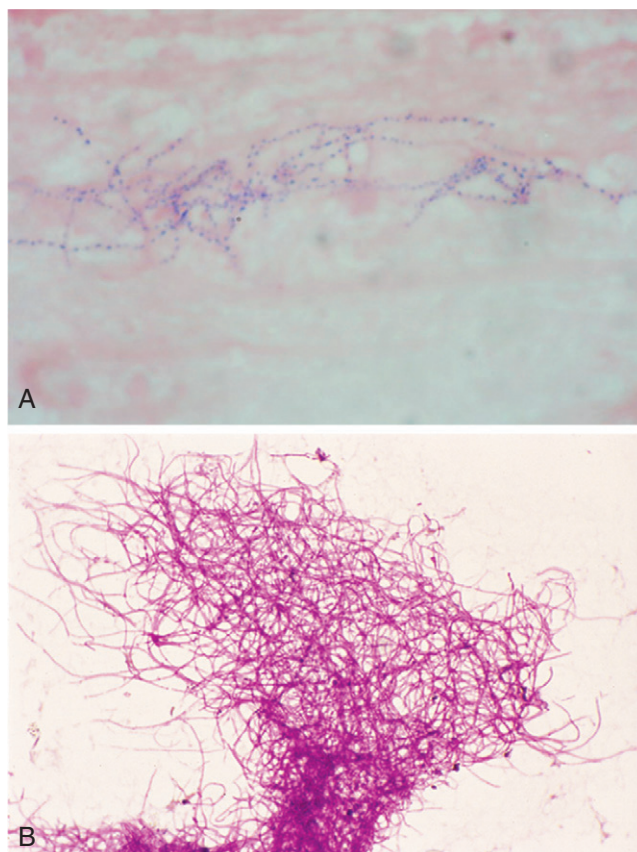


Fig. 16.14 A, Gram stained smear of *Nocardia* demonstrating irregular staining (×1000). B, Acid-fast staining of *Nocardia* showing partially acid-fast appearance (×1000). (Courtesy Steve Mahlen and Amanda Harrington.)

However, reports of infection in patients with no apparent illness or immunosuppressive therapy are increasing.

The taxonomy of the genus is evolving and has expanded the number of species recognized as human pathogens. There are about 100 named *Nocardia* spp., and more than one half have reportedly been isolated from humans. The most commonly encountered species are *Nocardia brasiliensis*, *Nocardia cyriacigeorgica*, *Nocardia farcinica*, *Nocardia abscessus* complex, and *Nocardia nova*. Less commonly encountered species include *Nocardia otitidiscaviarum*, *Nocardia pseudobrasiliensis*, *Nocardia paucivorans*, *Nocardia africana*, and *Nocardia transvalensis*. At one time, *Nocardia asteroides* was considered the most prominent *Nocardia* human pathogen. With the more frequent use of molecular methods for identification, it has since been found that *N. asteroides* is a rare human pathogen.

Virulence factors

The role of such factors as toxins and extracellular proteins in nocardiosis is unclear. No virulence factors have been identified, although virulence has been correlated with alterations in the components of the cell wall. The precise role of the various cell wall molecules in virulence is unknown. *Nocardia* spp. produce superoxide dismutase and catalase, which may provide resistance to oxidative killing by phagocytes. They also produce an iron-chelating compound called *nocobactin*. A correlation has been reported between the amount of nocobactin produced by the organism and its virulence.

Clinical infections

Infection occurs by two routes: pulmonary and cutaneous. Pulmonary infection by *Nocardia* occurs from the inhalation of the organism present in dust or soil and is the most common manifestation of disease. The disease appears to be associated with impaired host defenses because most individuals with *Nocardia* infections have an underlying disease or compromised immune system. Approximately 10% of *Nocardia* infections occur in seemingly healthy patients with no obvious immune impairment. Infection with *Nocardia* spp. can be serious. Approximately 40% of the diagnoses are made at autopsy. The mortality rate is high, and patients who survive often have significant tissue damage. Disseminated infections resulting in brain abscesses have been reported.

Pulmonary infections

The most common manifestation of infection is a confluent bronchopneumonia that is usually chronic but may be acute or relapsing. The disease generally progresses more rapidly than tuberculosis, and the course is measured in months rather than years. In the acute form, which is often seen in patients with underlying immune defects, the course is a matter of weeks.

The initial lesion in the lung is often a focus of pneumonitis that advances to necrosis. The abscesses that form can extend into the tissue and coalesce with each other. Extensive tissue involvement and damage result. In contrast with some pneumonias, there is little inflammatory response or scarring, there is no encapsulation of the abscesses, and no granuloma formation occurs. Sputum is thick and purulent. In contrast with infection by the anaerobic actinomycetes, no sulfur granules (masses of filamentous organisms bound together

by calcium phosphate) develop, and no sinus tract formation occurs. Dissemination to other organs, especially the brain, may occur, with reports of involvement of virtually every organ.

Cutaneous infections

Cutaneous infection occurs after inoculation of the organism into the skin or subcutaneous tissues usually seen in the hands and feet as a result of outdoor activity. The trauma most likely is minor, such as from a thorn or wood sliver.

The infection begins as a localized subcutaneous abscess that is invasive and quite destructive of the tissues and underlying bone. These lesions are termed **actinomycotic mycetomas**. *N. brasiliensis* is the most common cause of actinomycotic mycetoma. Some species of fungi also cause mycetomas; mycetomas caused by bacteria are called *actinomycotic mycetomas*, whereas mycetomas caused by fungi are known as **eumycotic mycetomas**. Depending on the causative agent, mycetomas are characterized by swelling, draining sinuses, and granules. About one half of the mycetomas seen clinically are caused by actinomycetes, and the remaining one half are caused by fungi. As the infection progresses, burrowing sinuses open to the skin surface and drain pus. The pus may be pigmented and contain "sulfur granules" (Fig. 16.15). The granules often appear yellow or orange and have a distinct granular appearance—hence the term *sulfur granules*.

Laboratory diagnosis

Microscopy

The gram-positive, beaded, branching filaments characteristic of *Nocardia* are often seen in sputum and in exudates or aspirates from skin or abscesses. The specimen often contains coccobacillary bodies as well. The beaded appearance of *Nocardia* may be confused as chains of gram-positive cocci; however, the beads do not usually touch each other and are not as regular as cocci. Presumptive identification of *Nocardia* can be made based on observation of a filamentous, branching isolate that is partially acid fast on staining with carbolfuchsin and decolorizing with a weak acid (0.5% to 1% sulfuric acid) compared with 3% hydrogen chloride in the stain for mycobacteria.

Wet mounts can also be performed on clinical specimens. Tissue and pus from the draining sinuses are the specimens of choice for direct examination. Granules may be seen in specimens from cutaneous infection. The small granules (80 µm to 130 µm) can be visualized by separating them from the pus with an inoculating needle and then washing in sterile saline. The granules of *N. cyriacigeorgica*, *N. farcinica*, *N. brasiliensis*, and *N. otitidiscaviarum* are soft and white to cream in color. They can be crushed between two glass slides to visualize the branching and cellular morphology, which comprises gram-positive, thin (0.5 to 1.0 µm in diameter), interwoven filaments. The granules may also be used to inoculate the appropriate growth media. The granules of a eumycotic mycetoma are composed of broad, interwoven, septate hyphae that are wider (2 to 5 µm) than those of actinomycotic mycetoma.

Culture characteristics

The growth requirements of *Nocardia* spp. are not as well defined as the growth requirements of many other medically important bacteria. These organisms show an oxidative-type

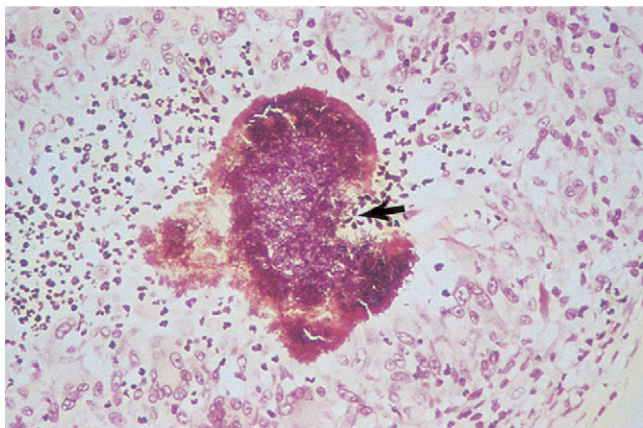


Fig. 16.15 Appearance of a sulfur granule collected from draining sinus tracts. These granules contain masses of filamentous organisms with pus materials. The arrow points to eosinophilic projections (clubs) characteristic of sulfur granules from gram-positive bacteria. (Hematoxylin and eosin stain, $\times 1000$.)

metabolism, and as a genus, they use a wide variety of carbohydrates. They do not require specific growth factors as do *Haemophilus* and *Francisella* spp. *Nocardia* spp. grow well on most common nonselective laboratory media incubated at temperatures between 22° and 37° C, although 3 to 6 days or more may pass before growth is seen. *Nocardia* spp. can be recovered on simple media containing a single organic molecule as a source of carbon. Media containing antimicrobial agents used for isolating fungi should not be used because *Nocardia* spp. are susceptible to many of the agents used in these media. Selective media, such as modified Thayer-Martin agar, may enhance recovery of *Nocardia* spp. by inhibiting the growth of contaminating organisms. *Nocardia* spp. grow on nonselective buffered charcoal–yeast extract agar.

Colonies of *Nocardia* spp. might have a chalky, matte, velvety, or powdery appearance and may be white, yellow, pink, orange, peach, tan, or gray pigmented. They can have a dry, crumbly appearance similar to breadcrumbs (Fig. 16.16). Table 16.5 outlines the colony appearance and tentative identification and differentiation of aerobic actinomycetes. Examination of colonies with a dissecting microscope may reveal the presence of aerial hyphae. These macroscopic and microscopic phenotypic colony morphologies provide the first clues to the identity of the organism as belonging to the genus *Nocardia*.

Identification

An isolate showing beaded, branching filaments that are gram positive and partially acid fast should be suspected to belong to the genus *Nocardia* (see Fig. 16.14). Phenotypic tests have been used to identify clinically relevant *Nocardia* spp. Methods employed for identification include (1) substrate hydrolysis (casein, tyrosine, xanthine, and hypoxanthine); (2) other substrate and carbohydrate use, arylsulfatase, and gelatin liquefaction; (3) antimicrobial susceptibility profile; and (4) fatty acid analysis by high-performance liquid chromatography. MALDI-TOF mass spectrometry is an emerging technology for the identification of *Nocardia* species. However, the most reliable method for the identification of *Nocardia* spp. is 16S rRNA gene sequencing, which is beyond the capabilities of most clinical laboratories. If routine tests used do not result in identification, the identity can be confirmed by a



Fig. 16.16 Colony morphology of *Nocardia* on chocolate agar. (Courtesy Steve Mahlen and Amanda Harrington.)

reference laboratory with experience in identification of such organisms.

Treatment

Treatment of nocardiosis often involves drainage and surgery along with administration of antimicrobials. The organisms are resistant to penicillin but susceptible to sulfonamides, although susceptibility profiles differ among different species. Antifungal agents have no activity against *Nocardia* spp. This fact underscores the importance of laboratory diagnosis because many of the clinical manifestations of pulmonary and cutaneous infection are shared with other organisms, including fungi. *Nocardia* spp. represent a classic example of a situation in which laboratory results are essential for proper antimicrobial treatment.

Other actinomycetes

Actinomadura

There are more than 60 species in the genus *Actinomadura*. The aerobic actinomycetes of clinical importance belonging to this genus include *A. madurae* and *A. pelletieri*. They cause mycetomas that are identical to those caused by *Nocardia*. The microscopic morphology and colony morphology of *Actinomadura* spp. are very similar to those of *Nocardia* spp. (see Table 16.5), although unlike *Nocardia* spp., which are partially acid-fast, *Actinomadura* spp. are not. The organisms can be differentiated using metabolic variations. *A. madurae* is cellobiose and xylose positive, whereas *Nocardia* spp. do not produce acid from these two carbohydrates. Treatment parallels that for infections with *Nocardia*.

Streptomyces

The genus *Streptomyces* comprises a large diverse group of bacteria. *Streptomyces* spp. are primarily saprophytes found as soil inhabitants and resemble other aerobic actinomycetes with regard to morphology and the diseases they cause. *Streptomyces somaliensis* is an established human pathogen associated with actinomycotic mycetoma in many countries. Many other *Streptomyces* spp. have been implicated

Table 16.5 Gram stain morphology, colony appearance, and preliminary grouping of aerobic actinomycetes

Genus	Gram stain morphology	Colony appearance on routine agar	Partially acid fast	Nitrate	Urea	Lysozyme resistance
<i>Actinomadura</i>	Moderate, fine, inter-twining, branching with short chains or spores, fragmentation	White-to-pink pigment, mucoid, molar tooth appearance after 2 weeks' incubation; sparse aerial hyphae	–	+	–	–
<i>Gordonia</i>	Short rods	Somewhat pigmented; <i>G. sputi</i> smooth, mucoid, and adherent; <i>G. bronchialis</i> dry and raised	±	+	+	–
<i>Mycobacterium</i>	Refractile or nonvisible	Dry, buff-colored; some species are pigmented; smooth or rough colonies	Strongly acid fast	±	±	Not performed
<i>Nocardia</i>	Branching, fine, intertwining, delicate filaments with fragmentation	Extremely variable; some isolates are β -hemolytic on SBA; wrinkled; often dry, chalky white appearance to orange-tan pigment; crumbly; may produce spores from aerial hyphae	+	+	+	+
<i>Rhodococcus</i>	Diphtheroid-like with minimal branching or coccobacillary; colony growth appears as coccobacilli in "zigzag" configuration	Nonhemolytic; round; often mucoid with orange-to-red, salmon-pink pigment developing within 4–7 days; pigment may vary widely	±	±	±	±
<i>Streptomyces</i>	Extensive branching with chains and spores; does not fragment easily	Glabrous or waxy heaped colonies; variable morphology; wide range of pigmentation from cream to brown-black; white aerial hyphae	–	±	±	–
<i>Tsukamurella</i>	Mostly long bacilli that fragment; no spores or aerial hyphae	May have rhizoid edges, dry, white-to-creamy-to-orange	±	–	+	+

+, Predominantly positive; –, predominantly negative; ±, predominantly positive with some negative isolates; SBA, sheep blood agar.
 Modified from Conville P. S., & Witebsky, F. G. (2015). *Nocardia*, *Rhodococcus*, *Gordonia*, *Actinomadura*, *Streptomyces*, and other aerobic actinomycetes. In J. H. Jorgensen, et al. (Eds.), *Manual of clinical microbiology* (11th ed.). Washington, DC: ASM Press.

in different types of human infections. See Table 16.5 for a comparison of the colony morphology and metabolic characteristics of *Streptomyces* spp. with those of other aerobic actinomycetes. Isolates from this genus may need to be identified by reference laboratories.

Gordonia

Members of the genus *Gordonia* are aerobic, catalase positive, gram positive to gram variable, partially acid fast, and non-motile. They grow with mycelial forms that fragment into rod-shaped or coccoid elements—hence the term *nocardioform*. Members are distinguished from similar organisms by simple biochemical tests. They differ from rapidly growing mycobacteria by their partial acid fastness and the absence of arylsulfatase. They are distinguished from the genus *Nocardia* by their ability to reduce nitrate and the absence of mycelia.

Gordonia spp. are typically isolated from environmental sources. So far, reports of human infections are uncommon but increasing recently. In almost all cases, patients were immunosuppressed as a result of underlying diseases, and infections by *Gordonia* spp. occurred only secondarily. Most reported cases of infections were caused by *Gordonia bronchialis* and included postsurgical sternal wounds, coronary artery infection, and infection from central venous catheters. Infections by *Gordonia* spp. might be underreported because

the isolates are considered insignificant or misidentified as *Nocardia* or *Rhodococcus*. *Gordonia* spp. are susceptible to several antimicrobial agents, including many β -lactams, quinolones, aminoglycosides, macrolides, and other agents active against gram-positive organisms. In the absence of clear guidelines, treatment should be based on susceptibility test results.

Rhodococcus

Rhodococcus equi, the most common human isolate in this genus, is found in soil and causes respiratory tract infections in animals. Human infection is rare, although an increased incidence in immunosuppressed patients, particularly those with acquired immunodeficiency syndrome, has been reported. Contact with farm animals and feces is an important risk factor. Lung infections account for about 80% of human disease in immunosuppressed patients.

On Gram-stained smear, *R. equi* may demonstrate filaments, some with branching. *R. equi* may be partially acid fast or acid fast. On SBA, the colonies resemble *Klebsiella* spp. and can form a salmon-pink pigment on prolonged incubation, especially at room temperature. Biochemical identification is difficult because it does not ferment carbohydrates and shows a variable reaction to many characteristics (e.g., nitrate reduction, urease). Key features for the identification

of *Rhodococcus* is the salmon-pink pigment and Gram stain morphology showing characteristic diphtheroid gram-positive rods with traces of branching.

Tropheryma whipplei

Tropheryma whipplei is the agent of **Whipple disease**. *T. whipplei* is a facultative intracellular pathogen first identified in 1991 by using PCR from a duodenal biopsy specimen. Phylogenetic analysis has revealed that *T. whipplei* is a gram-positive actinomycete, most closely related to the genera *Rothia*, *Rhodococcus*, *Arthrobacter*, and *Dermatophilus*. *T. whipplei* has been detected in sewage and in human feces, saliva, and gastric secretions and is ubiquitous in the environment. Whipple disease was first described in 1907 by George H. Whipple and was first successfully treated in 1952 with antimicrobials. If untreated, this is a uniformly fatal disease, with typical symptoms of diarrhea, weight loss, malabsorption, arthralgia, and abdominal pain. Other organs may also be involved, including the CNS and the heart. *T. whipplei* has been associated with several cases of culture-negative endocarditis.

Despite a high incidence of human colonization, Whipple disease is rare and is seen most commonly in **White middle-age men with European heritage**. It is believed that asymptomatic carriage or a mild self-limiting gastroenteritis occurs in many children following ingestion of the organism. Cell- and humoral-mediated immunity develops, and the bacteria are cleared. In some individuals, the infection persists over a period of many years and spreads systemically, producing the classic Whipple disease.

Diagnosis is best made by microscopic examination of endoscopic biopsy specimens. The presence of characteristic periodic acid–Schiff staining is strongly suggestive of Whipple disease. In addition, rod-shaped bacteria can be observed in macrophages from infected tissues. The bacteria do not Gram stain well and are acid-fast negative. The organism has been cultivated in stable cell lines and has been adapted to an axenic culture medium supplemented with essential amino acids; however, these techniques are not easily performed in the clinical microbiology laboratory. *T. whipplei* can be identified with PCR or 16S rRNA gene sequencing. Treatment depends on disease presentation (classic Whipple disease, CNS involvement, endocarditis). *T. whipplei* is usually susceptible to trimethoprim-sulfamethoxazole, ceftriaxone and other β -lactams, doxycycline, chloramphenicol, rifampin, and aminoglycosides.

Spore-forming, nonbranching, catalase-positive bacilli

Bacillus

General characteristics

Members of the genus *Bacillus* stain gram positive or gram variable and are aerobic or facultative anaerobic bacilli that form endospores. There are more than 90 species within the genus, and all are widely distributed in the soil and the environment. Members of the genus *Bacillus* are metabolically

diverse, and some species are thermophiles that grow best at 55° C or higher. The survival of *Bacillus* spp. in nature is aided by the formation of spores that are resistant to conditions to which vegetative cells are intolerant.

Most species grow well on SBA and other commonly used enriched media but typically do not grow on Columbia colistin-naladixic acid agar. Colony characteristics differ considerably among the species and are often influenced by the type of medium used. Most species form nonpigmented colonies and are catalase positive. Members of the genus *Bacillus* can be confused with aerotolerant strains of the other primary endospore-forming genus, *Clostridium*. *Clostridium* spp. are typically catalase negative, although some strains may form trace amounts. In addition, *Bacillus* spp. form endospores aerobically and anaerobically, whereas *Clostridium* spp. form endospores anaerobically only. *Bacillus* spp. are divided into groups based on genetic identity and morphologic features. The *Bacillus cereus* group, consisting of *B. anthracis*, *B. cereus*, *B. thuringiensis*, *B. mycoides*, *B. pseudomycoides*, and *B. cytotoxicus*, among others, is the most medically relevant group. The species in the *B. cereus* group are genetically very closely related.

Many *Bacillus* spp. are commonly isolated and often regarded as laboratory contaminants. Therefore most laboratories do not identify the majority of *Bacillus* isolates to the species level. Several species are considered insect and plant pathogens, and some species are associated with human infections, including *B. anthracis* and *B. cereus*. Koch, in the development of Koch's postulates, showed that *B. anthracis* caused **anthrax** in cattle and helped prove the germ theory of disease. Historically, *B. anthracis* has been the most important member of this genus; however, anthrax is rarely reported in the United States. Because *B. anthracis* is considered to be a potential bioterrorism agent, it is important for clinical laboratories to rule out this organism when *Bacillus* spp. are isolated and to send isolates to a reference laboratory in the Laboratory Response Network (LRN) when it cannot be ruled out.

In 2016, *B. cereus* biovar *anthracis* was added to the list of potential bioterrorism agents. Currently, this biovar has only been found in African countries (including Cameroon, Côte d'Ivoire, Liberia, Democratic Republic of Congo, and Central African Republic) and is known to cause anthrax-like disease in gorillas, chimpanzees, elephants, mongooses, and other animals. To date, there have been no known cases of human infection caused by *B. cereus* biovar *anthracis*. *B. cereus* biovar *anthracis* produces all of the virulence factors that *B. anthracis* produces, including capsule, **protective antigen (PA)**, **edema factor (EF)**, and **lethal factor (LF)**.

Bacillus anthracis

Virulence factors

The virulence of *B. anthracis* depends on a glutamic acid capsule and a three-component protein exotoxin. The genes that code for the toxin and the enzymes responsible for capsule production are carried on plasmids. If a virulent isolate is repeatedly subcultured in vitro, the plasmids can be lost, and the organism becomes avirulent. The capsule, which protects the organism from phagocytosis, is a polypeptide of D-glutamic acid. This particular isomer of glutamic acid is resistant to hydrolysis by host proteolytic enzymes because it is

the “unnatural” form of the amino acid (L-glutamic acid). Although the capsule is necessary for virulence, antibodies against the capsule do not confer immunity.

Anthrax toxin consists of three proteins: PA, EF, and LF, each of which individually is nontoxic but together act synergistically to produce damaging effects. PA serves as a necessary binding molecule for EF and LF, permitting their attachment to specific receptors on the host cell’s surface. The effect of EF and LF is seen when either is combined with PA. Edema results from the combination of PA with EF, whereas death occurs when PA and LF combine. EF is an adenylate cyclase that increases the concentration of cyclic adenosine monophosphate (cAMP) in host cells. LF is a protease that kills host cells by disrupting the transduction of extracellular regulatory signals.

Clinical infections

Anthrax is a common disease in livestock worldwide when the vaccine is not used. The disease is not spread from animal to animal but rather is spread by animals feeding on plants contaminated with the spores or from contaminated soil. Humans are infected primarily as a result of accidental or occupational exposure to animals or animal products. Human anthrax in the United States is rare. In 2001, 11 cases of inhalation anthrax and 11 cases of cutaneous anthrax occurred in the United States. Investigations into these cases revealed that they were bioterrorism related, and a suspect in the case was eventually identified. Cases mostly occurred among postal workers as a result of exposure to spore-tainted material (powder in or on envelopes) sent through the mail, although the actual source remains unknown for some cases. *B. anthracis* as an agent of bioterrorism is discussed in [Chapter 30](#). From 2002 to 2019, there were nine cases of anthrax in the United States, none of which were attributed to bioterrorism events. Every year, there are outbreaks of anthrax in livestock in the United States, usually in western states in animals that were not vaccinated.

Worldwide, cases of anthrax number several thousand per year. The disease is enzootic in many parts of the world, including Africa, Central America, and South America. The largest outbreak involving primarily cutaneous anthrax occurred in Zimbabwe in the early 1980s with approximately 10,000 cases. Numerous names have been given to infections with *B. anthracis*. Most of these refer to occupational associations. Terms such as *wool sorter’s disease* and *ragpicker’s disease* were used to describe infection with the spores of *B. anthracis* as a result of handling contaminated animal fibers, hides, and other animal products. Three main forms of anthrax are recognized in humans: cutaneous, inhalation or pulmonary, and gastrointestinal. Infection results from wound contamination, inhalation, or ingestion of spores, which germinate within the host tissue. A fourth form, called *injectional anthrax*, has emerged more recently and is recognized as an additional form of clinical infection. Infection results from direct injection of spores into tissue generally during the administration of drugs of abuse.

Cutaneous anthrax

Cutaneous anthrax can occur when wounds are contaminated with anthrax spores acquired through skin cuts, abrasions, or insect bites. About 99% of anthrax cases in the world are the cutaneous type. Most commonly, cutaneous anthrax occurs on the hands, arms, neck, and head. In this form of anthrax, a

small pimple or papule appears at the site of inoculation 2 to 3 days after exposure. A ring of vesicles develops, and the vesicles coalesce to form an erythematous ring. A small dark area appears in the center of the ring and eventually ulcerates and dries, forming a depressed black necrotic central area known as an **eschar** or *black eschar*. The lesion is sometimes referred to as a *malignant pustule*, even though it is not a pustule and is not malignant. The lesion is painless and does not produce pus unless it becomes secondarily infected with a pyogenic organism. The eschar is normally 1 to 3 cm in diameter, but it may be more extensive. The eschar begins to heal after 1 to 2 weeks. The lesion dries, separates from the underlying base, and falls off, leaving a scar. Usually, the infection remains localized, but regional lymphangitis and lymphadenopathy appear. If septicemia occurs, symptoms of fever, malaise, and headache are seen. Normally, in uncomplicated cases, no systemic symptoms are present. The mortality rate of cutaneous anthrax is 20% with no treatment. However, those who are treated usually survive. Cutaneous anthrax is the most common and has the lowest mortality.

Inhalation anthrax

Inhalation anthrax, also called **wool sorter’s disease**, is acquired when spores are inhaled into the pulmonary parenchyma. The infection begins as a nonspecific illness consisting of mild fever, fatigue, and malaise 2 to 5 days after exposure to the spores. It resembles an upper respiratory tract infection, such as that seen with colds and flu. This initial, mild form of the disease lasts 2 to 3 days. It is followed by a sudden severe phase in which respiratory distress is common. The severe phase of the disease has a high mortality rate; inhalation anthrax is nearly always fatal if not treated. The respiratory problems (dyspnea, cyanosis, pleural effusion) are followed by disorientation, coma, and death. The course of the severe phase (onset of respiratory symptoms to death) may last only 24 hours. With proper treatment, approximately 55% of patients with inhalation anthrax will survive.

Gastrointestinal anthrax

Gastrointestinal anthrax occurs when the spores are inoculated into a lesion on the intestinal mucosa after ingestion of undercooked or raw meat containing *B. anthracis* spores. The symptoms of gastrointestinal anthrax include abdominal pain, nausea, anorexia, and vomiting. Bloody diarrhea can also occur. Because this form of the disease is difficult to diagnose, the fatality rate is about 50% with no treatment. Gastrointestinal anthrax accounts for less than 1% of total cases worldwide; a case of gastrointestinal anthrax occurred in the United States in 2009 involving a woman who participated in a drumming circle. The drums were made with animal hides.

Injectional anthrax

Injectional anthrax is characterized by soft tissue infection associated with “skin popping” or other forms of injection drug use and results from the direct injection of the spores into tissue. Although it was initially reported in 2000, injectional anthrax was first recognized in Scotland during a 2009 to 2010 outbreak associated with heroin. There were 47 confirmed cases and 13 deaths, and 7 cases and 5 deaths were additionally identified in England and Germany. Injectional anthrax can be associated with necrotizing fasciitis, organ failure, shock, coma, and meningitis, and it has a much

higher rate of mortality than cutaneous anthrax. Injectional anthrax soft tissue infections have not been associated with black eschar formation. Lack of eschar, severity of disease, and increased mortality rate make this form clinically distinct from the cutaneous form. To date, there have been no cases of injectional anthrax in the United States.

Complications

Approximately 5% of patients with anthrax (cutaneous, inhalation, gastrointestinal, or injectional) develop meningitis, with a greater proportion of cases occurring in the inhalation and injectional forms. The symptoms are typical of any bacterial meningitis and develop rapidly. Unconsciousness and death, if they occur, follow 1 to 6 days after initial exposure. Evaluation of cases suggests that initial therapy should be a multidrug regimen, including a fluoroquinolone and one or more additional agents with good CNS penetration. Recovery from infection appears to confer immunity.

An effective vaccine is available for individuals who are at risk for occupational exposure. This includes veterinarians, laboratorians working with *B. anthracis*, and persons in the military because of the biological warfare potential. The human vaccine is a cell-free culture filtrate of bacterial proteins adsorbed onto an aluminum salt. The vaccine was approved in 2015 by the U.S. Food and Drug Administration (FDA) for anyone who may have been exposed to anthrax. As described earlier, vaccines are also available for use in farm animals.

Laboratory diagnosis

Microscopy

B. anthracis is a large (1.0 to 1.5 μm $\times$ 3.0 to 5.0 μm), square-ended, gram-positive or gram-variable rod found singly or in chains (Fig. 16.17). When in chains, the ends of the single cells fit snugly together; this, together with the unstained central spore, gives the appearance of bamboo rods. Young cultures stain gram positive; as the cells age, or if they are under nutritional stress, they become gram variable. In Gram stain preparations of clinical samples, vegetative cells can appear with clear zones around the cells, representing the presence of a capsule. The presence of large encapsulated gram-positive rods in blood is strongly presumptive for *B. anthracis* identification. As the bacteria are subcultured, capsule production ceases. Incubation in an atmosphere containing increased CO_2 can stimulate capsule production. Spores are generally not present in clinical samples but sometimes can be seen as unstained areas within the cells. Spores can be observed with a spore stain. With this technique, vegetative cells stain red, and the spores stain green (Fig. 16.18).

Cultural characteristics

On SBA, colonies of *B. anthracis* are nonhemolytic, large (2 to 5 mm), gray, and flat with an irregular margin because of outgrowths of long, filamentous projections of bacteria that can be seen with a dissecting microscope. The term **Medusa head** has been used to describe the colony morphology of *B. anthracis*. Colonies have a tenacious consistency, holding tightly to the agar surface, and when the edges are lifted with a loop, they stand upright without support. This has been described as having the appearance or characteristic of beaten egg whites. Although *B. anthracis* is nonhemolytic on SBA, weak hemolysis may appear under areas of heavy growth



Fig. 16.17 Gram stained smear of *Bacillus* ($\times 1000$). (Courtesy Cathy Bissonette.)

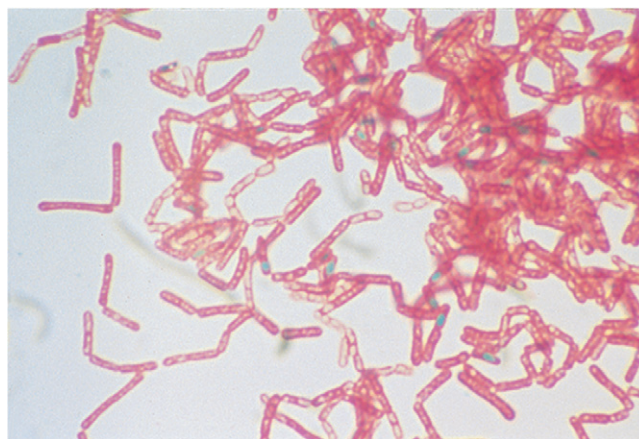


Fig. 16.18 Spore stain of *Bacillus*. Vegetative cells are red; spores are green ($\times 1000$). (Courtesy Cathy Bissonette.)

after prolonged incubation; this should not be confused with β -hemolysis because many *Bacillus* spp. other than *B. anthracis* are hemolytic.

Because *B. anthracis* would typically be isolated from normally sterile sites, such as blood, lung tissue, and CSF, selective media are not usually needed for recovery. Although some strains of *B. anthracis* grow on phenylethyl alcohol (PEA) medium, growth is usually weak. The current American Society of Microbiology (ASM) sentinel level testing protocol for the presumptive identification of anthrax recommends using SBA and MacConkey (MAC) agar (or eosin-methylene blue [EMB] agar) for cutaneous, tissue, and stool specimens. For respiratory specimens, SBA, chocolate (CHOC) agar, and MAC agar (or EMB agar) are recommended, whereas for CSF cultures, SBA, CHOC agar, and broth medium, such as Tryptic soy broth (TSB), should be used.

Identification

Caution should always be used when working with an isolate suspected of being *B. anthracis*. Work should be performed in a biological safety cabinet, and the area should be disinfected when the work is completed. Approved tests that should be performed by LRN sentinel laboratories include Gram staining and colony morphology, catalase, and motility tests. Gram staining can be performed directly on clinical specimens or

on culture isolates. If the microscopic and colony morphologies of the isolate are compatible with *B. anthracis*, additional tests need to be performed, typically at an LRN reference laboratory.

B. anthracis is catalase positive and grows aerobically or anaerobically. Although *B. anthracis* ferments glucose, it fails to ferment mannitol, arabinose, or xylose. *B. anthracis* produces lecithinase; an opaque zone can be seen around colonies growing on egg-yolk agar. This species grows in high-salt (7% sodium chloride) and low pH (<6) conditions. In contrast with *B. cereus*, *B. anthracis* is generally susceptible to penicillin (10 U/mL). Characteristics important to differentiate *B. anthracis* from the closely related *B. cereus* are listed in Table 16.6.

B. anthracis is nonmotile, distinguishing it from most other members of the genus; *B. mycoides* is also nonmotile. Motility should be tested by inoculation into semi-solid medium. Capsule production by *B. anthracis* can be detected by India ink staining on blood or CSF specimens or on cells isolated in media supplemented with sodium bicarbonate, although this technique is not routinely performed by clinical laboratories.

If the laboratory cannot rule out *B. anthracis*, the isolate is sent to an LRN reference laboratory, usually a state laboratory, for confirmation. The reference laboratory is likely to perform direct fluorescent antibody assays for a cell wall polysaccharide and a capsule antigen. The presence of both antigens is confirmation for *B. anthracis*. Antimicrobial susceptibility testing is performed by reference or national laboratories. The ASM sentinel level anthrax protocol does not recommend using MALDI-TOF mass spectrometry or other automated systems for the identification of *B. anthracis* for biosafety reasons and because of the potential for misidentification. Nucleic acid amplification tests have been developed, but such applications for diagnosis and identification of *B. anthracis* are only used in LRN reference or national level laboratories.

B. cereus biovar *anthracis* strains are usually nonhemolytic on SBA, like *B. anthracis*, but are motile, unlike *B. anthracis*. The exception is goat strains from the Democratic Republic of the Congo, which are nonmotile. As described earlier, there have been no known human cases of anthrax-like disease caused by *B. cereus* biovar *anthracis* to date.

Table 16.6 Differentiation of *Bacillus anthracis* and *Bacillus cereus*

Characteristic	<i>B. anthracis</i> ^a	<i>B. cereus</i> ^a
Hemolysis on SBA	–	+
Motility	–	+
Penicillin susceptibility	S	R
Lecithinase production	+	+
Fermentation of salicin	–	+/-
Growth in penicillin (10 U/mL) agar	–	+
Gelatin hydrolysis	–	+
Growth on phenylethyl alcohol agar	–	+

^aAll cultures were incubated at 36° to 37° C.

R, Resistant; S, sensitive; SBA, sheep blood agar; +, >85% positive; –, 0% to 15% negative; +/-, 16% to 25% positive.

From Turenne, C. Y., & Alexander, D. C. (2019). *Bacillus* and other aerobic endospore-forming bacteria. In K. C. Carroll, et al. (Eds.). *Manual of clinical microbiology* (12th ed., p. 468). Washington, DC: ASM Press.

Treatment

Most isolates of *B. anthracis* are susceptible to penicillin, but resistance can occur in the absence of β -lactamase production. Penicillin should not be used alone in treatment. The organism is often susceptible to many broad-spectrum antimicrobial agents, including carbapenems, tetracyclines, clindamycin, erythromycin, linezolid, fluoroquinolones, and chloramphenicol. Current recommendations for antimicrobial therapy depend on the type of anthrax and whether or not the illness is systemic. Ciprofloxacin is the primary recommended therapy in most cases, either alone or in combination with other agents. Postexposure prophylaxis recommendations for inhalational anthrax depend on the status of the exposed individual (children, nonpregnant adults, pregnant women/nursing mothers) but usually includes either ciprofloxacin or doxycycline and vaccination.

Bacillus cereus

B. cereus is a relatively common cause of food poisoning and opportunistic infections in susceptible hosts. Food poisoning caused by *B. cereus* takes two forms: diarrheal and emetic. The diarrheal syndrome, usually associated with ingestion of meat or poultry, vegetables, and pastas, is characterized by an incubation period of 8 to 16 hours. Affected individuals have abdominal pain and diarrhea. About 25% of individuals have vomiting; fever is uncommon. The average duration of the illness is 24 hours. The diarrheal form is clinically indistinguishable from diarrhea caused by *Clostridium perfringens*.

The emetic form has the predominant symptoms of nausea and vomiting 1 to 5 hours after ingestion of contaminated food. Diarrhea is present in about one third of affected individuals. This form has been associated particularly with ingestion of fried rice prepared in Asian restaurants. The average duration of the illness is 9 hours. For both the diarrheal and the emetic forms of *B. cereus* food poisoning, the illness is usually mild and self-limiting. The two forms of illness are caused by two distinct enterotoxins. The enterotoxins are compared in Table 16.7.

Culture of suspected food from a food poisoning incident may be performed to isolate and quantify *B. cereus*. If more than 10^5 *B. cereus* cells per gram of food are present and other pathogens are absent, food poisoning by this organism is confirmed. Because *B. cereus* can be found in small numbers in a significant proportion of healthy people, quantitative cultures must be done. To confirm the organism as the cause of the disease, viable counts from the stool should also be at least 10^5 cells per gram. Most food poisoning cases caused by *B. cereus* do not require antimicrobial treatment. However, treatment may be indicated in other *B. cereus* infections. In contrast with *B. anthracis*, *B. cereus* is resistant to penicillin and all of the other β -lactam antibiotics except the carbapenems. Treatment with vancomycin or clindamycin with or without an aminoglycoside has been successful.

B. cereus is similar to *B. anthracis* in many ways—morphologically and metabolically. Differentiation of the two species is outlined in Table 16.6. *B. cereus* can be grown aerobically at 37° C on SBA. A β -hemolytic frosted glass–appearing colony (Fig. 16.19) containing spore-forming, gram-positive bacilli that are motile, able to ferment salicin, and lecithinase positive is likely *B. cereus*. *B. cereus* is biochemically

Table 16.7 Comparison of enterotoxins produced by *Bacillus cereus*

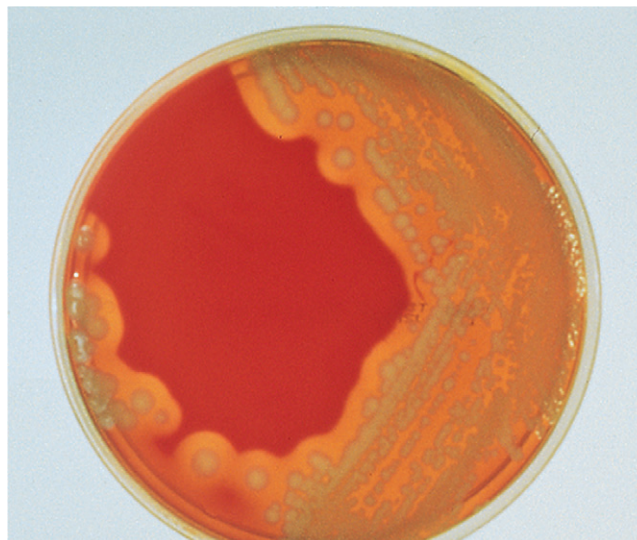
Characteristic	Type of enterotoxin	
	Diarrheal	Emetic
Clinical syndrome		
Incubation period	8–16 hours	1–5 hours
Diarrhea	Very common	Fairly common
Vomiting	Occasional	Very common
Duration of illness	12–24 hours	6–24 hours
Foods implicated	Meat products, soups, vegetables, puddings, sauces	Fried or boiled rice
Enterotoxins		
Molecular weight	≈50,000	<5000
Stability to heat	–	+
Stimulation of adenylate cyclase–cAMP system in intestinal epithelial cells	+	–
cAMP, Cyclic adenosine monophosphate. Modified from Braude, A. I., et al. (Eds.). (1986). <i>Infectious diseases and medical microbiology</i> (2nd ed.). Philadelphia: Saunders.		

identical to *B. thuringiensis* except that *B. thuringiensis*, an insect pathogen, typically produces parasporal crystals that can be observed by using phase-contrast microscopy or spore staining.

B. cereus eye infections are the most common type of non-gastrointestinal infection caused by this organism; these include endophthalmitis, panophthalmitis, and keratitis with abscess formation. *B. cereus* infection in penetrating ocular trauma cases is nearly always associated with a poor visual outcome. This organism has also been documented as a cause of meningitis, septicemia, endocarditis, osteomyelitis, and many other types of infections. Although rare, these serious, nongastrointestinal infections occur more frequently in intravenous drug abusers, neonates, and immunosuppressed and postsurgical patients. In addition, there have been a few reports of *B. cereus* strains carrying the *B. anthracis* toxin genes. In these cases, *B. cereus* caused severe pneumonia clinically similar to pulmonary anthrax. In 2010 and 2011, *B. cereus* was reported in nonsterile alcohol pads used as an antiseptic measure before injections. The bacteria resulted in several fatal infections, leading to the recall of millions of cases of pads and the questioning of the use of such products. Most alcohol pads used in hospitals are sterile.

Other *Bacillus* species

Infections by other members of the genus *Bacillus* are rare. These include, but are not limited to, *B. subtilis*, *B. licheniformis*, *B. circulans* (now *Niallia circulans*), *B. pumilus*, and *B. sphaericus* (now *Lysinibacillus sphaericus*). These organisms have been reported to cause food poisoning, bacteremia, meningitis, pneumonia, and other infections. However, they are more commonly seen as contaminants.

Fig. 16.19 *Bacillus cereus* on sheep blood agar. (Courtesy Cathy Bissonette.)

POINTS TO REMEMBER

- *C. diphtheriae* causes serious disease in populations of countries where the diphtheria vaccine is not available.
- Nondiphtheria *Corynebacterium* species are opportunistic pathogens.
- *L. monocytogenes* can be differentiated from streptococci and enterococci on the basis of Gram stain morphology, catalase reaction, and motility.
- *E. rhusiopathiae* differs from *L. monocytogenes* in catalase reaction, hydrogen sulfide production, and lack of ability to grow at 4°C.
- *A. haemolyticum* can be differentiated from other non-spore-forming gram-positive bacilli and β -hemolytic streptococci by Gram stain morphology, catalase activity, and the CAMP inhibition reaction or reverse CAMP test.
- *G. vaginalis* is part of the normal biota of the urogenital tract but might play a role in BV. It is weakly β -hemolytic on HBT agar and stains as a gram-variable rod.
- Aerobic actinomycetes are generally soil inhabitants. They are weak pathogens sometimes associated with wounds after traumatic implantation into subcutaneous tissue.
- *Nocardia* spp. are gram-positive, filamentous organisms that can grow on nutritionally simple media and are partially acid fast.
- To provide accurate identification and speciation of actinomycetes, 16S rRNA gene sequencing is often required; however, this is unavailable in most clinical laboratories.
- Members of the genus *Bacillus* are aerobic, gram-positive, catalase-positive, rod-shaped organisms that form endospores.
- Many aerobic, gram-positive spore-forming bacilli are rarely associated with human infections. The most important pathogen in this group is *B. anthracis*.
- *B. anthracis* is generally susceptible to penicillin and is nonmotile and nonhemolytic on SBA, features important to differentiate the organism from *B. cereus*.
- *B. cereus* is a common cause of food poisoning and opportunistic infections. Food poisoning caused by *B. cereus* occurs in two forms: diarrheal and emetic.

LEARNING ASSESSMENT QUESTIONS

1. An isolate with the appropriate colony and microscopic morphology may be suspected to be *Bacillus anthracis* if it has which of the following characteristics?
 - a. β -Hemolytic on SBA
 - b. Nonmotile
 - c. Catalase negative
 - d. Gram negative, non-spore forming
2. An aerobic, gram-positive, spore-forming bacillus was isolated from raw vegetables that were associated with an outbreak of gastroenteritis. The organism produced β -hemolysis, was catalase positive, and was motile. Which of the following is the most likely organism?
 - a. *Bacillus anthracis*
 - b. *Nocardia nova*
 - c. *Bacillus cereus*
 - d. *Tsukamurella* spp.
3. *Bacillus cereus* is most noted for causing which illness?
 - a. Food poisoning
 - b. Meningitis
 - c. Sexually transmitted disease
 - d. Urinary tract infection
4. Which forms of infection are caused by *Bacillus anthracis*?
 - a. Inhalation
 - b. Gastrointestinal
 - c. Cutaneous
 - d. All of the above
5. The functionality of lethal factor requires the presence of which other protein from *Bacillus anthracis* to form an active toxin?
 - a. Cyclic adenosine monophosphate
 - b. Edema factor
 - c. D-Glutamic acid
 - d. Protective antigen
6. Describe the appearance of spore-forming bacteria seen with the spore stain.
7. How do *Corynebacterium* species often appear on Gram-stained smears?
 - a. Pleomorphic, gram-positive, club-shaped bacilli that appear in palisades or in V and L formations
 - b. Branching gram-positive bacilli that appear as fine, intertwining, delicate filaments
 - c. Short, thin gram-positive bacilli that appear in chains
 - d. Large square-ended, gram-positive or gram-variable bacilli in chains where the ends of the single cells fit snugly together
8. Biochemical tests performed on a gram-positive bacillus were consistent with *Corynebacterium diphtheriae*. Which definitive test should the laboratory scientist perform next?
 - a. Prepare a smear of the isolate for Gram stain, and observe it for its pleomorphic morphology.
 - b. Prepare a methylene blue stain, and examine it for metachromatic granules.
 - c. Perform an Elek test to determine whether the organism produces exotoxin.
 - d. Subculture the organism to cystine-tellurite blood agar, and examine it for black colonies.
9. Why is diphtheria uncommon in the United States?
 - a. Elimination of the insect vector
 - b. Vaccination of the animal reservoir
 - c. Routine use of an effective human vaccine
 - d. Elimination of the bacteria with aggressive antimicrobial therapy
10. True infections with nondiphtheria *Corynebacterium* spp., such as *C. jeikeium* or *C. striatum*, often occur in immunocompromised patients or patients who have had which of the following?
 - a. Insertion of hardware or prosthetic devices
 - b. Coronary artery bypass surgery
 - c. Vitamin B₁₂ deficiency
 - d. A lengthy hospital stay
11. When recovered from urine samples, which test can help differentiate which clinically significant *Corynebacterium* spp.?
 - a. Gelatin hydrolysis; *C. ulcerans*
 - b. Reverse CAMP; *C. pseudotuberculosis*
 - c. Alkaline phosphatase; *C. amycolatum*
 - d. Urease; *C. urealyticum*
12. A newborn female patient becomes febrile and will not feed for about an hour after birth. A gram-positive rod is recovered from blood cultures from the newborn. The isolate has the following characteristics:
Weakly β -hemolytic on SBA
Gram-positive bacilli, no spores observed
Catalase positive
Hydrogen sulfide negative
Motile at room temperature
What is the most likely identity of the isolate?
 - a. *Erysipelothrix rhusiopathiae*
 - b. *Listeria monocytogenes*
 - c. *Corynebacterium ureilyticum*
 - d. *Gardnerella vaginalis*
13. A commercial fisherman with red sores on his hands was seen by his physician. Biopsy and culture of one of the lesions grew an organism with the following characteristics:
Nonhemolytic on SBA
Gram-positive bacilli, no spores observed
Catalase negative
Hydrogen sulfide production positive
Growth in gelatin resembled a test-tube brush
What is the most likely identification?
 - a. *Rhodococcus equi*
 - b. *Listeria monocytogenes*
 - c. *Lactobacillus acidophilus*
 - d. *Erysipelothrix rhusiopathiae*
14. A 42-year-old male patient from Guatemala cut his bare feet on thorns while walking. A subcutaneous abscess developed, and when the patient is seen by a physician, his foot is swollen. When the physician presses the wound, purulence is expressed along with some soft, white granules. A filamentous organism that is partially acid fast is recovered from the granules. This is most likely an _____ mycetoma caused by _____.
 - a. Actinomycotic; *Pseudallescheria boydii*
 - b. Actinomycotic; *Nocardia brasiliensis*
 - c. Eumycotic; *Madurella mycetomatis*
 - d. Eumycotic; *Gordonia bronchialis*

15. Which other organisms can give similar clinical and laboratory findings as those for *Listeria monocytogenes*? How are these organisms differentiated from *L. monocytogenes*?
16. A 17-year-old male patient presented to an emergency department with a history of multiple episodes of febrile pharyngitis followed in 10 to 14 days by extensive desquamation of his hands and feet. The recurrences have followed several courses of antimicrobial therapy, including amoxicillin and cephalosporins. Rapid group A streptococci screens and cultures have been consistently negative for *Streptococcus pyogenes*. A specimen with a request for an alternative agent is submitted to a reference laboratory, and the following results are observed:
SBA: small, slightly hemolytic colony, which at 48 hours is a dark spot sunken in the agar
Catalase negative
Nitrate negative
Reverse CAMP test positive
The patient was subsequently treated with erythromycin, and he recovered. What was the etiologic agent?
a. *Corynebacterium diphtheriae*
b. *Arcanobacterium haemolyticum*
c. *Listeria monocytogenes*
d. *Rhodococcus equi*
17. A 57-year-old male patient from New York City presents to the emergency department with diarrhea, arthralgia, abdominal pain, malabsorption, and weight loss of 10 lb (4.5 kg) over the past month. A duodenal biopsy is performed, but no infectious agent is recovered on culture media. However, on a Gram-stained smear, a gram-positive rod is observed in macrophages in the biopsy tissue. The organism is identified by 16S rRNA gene sequencing. What is the most likely identity of this organism?
a. *Bacillus anthracis*
b. *Nocardia asteroides*
c. *Tropheryma whippelii*
d. *Erysipelothrix rhusiopathiae*

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Neisseria species and *Moraxella catarrhalis*

Lauren Roberts

CHAPTER OUTLINE

General characteristics, 373

Pathogenic *Neisseria* species, 373

Virulence factors, 373

Neisseria gonorrhoeae, 374

Neisseria meningitidis, 380

Moraxella catarrhalis, 383

Clinical infections, 383

Laboratory diagnosis, 383

Commensal *Neisseria* species, 384

Identification, 384

Neisseria cinerea, 384

Neisseria flavescens, 387

Neisseria lactamica, 387

Neisseria mucosa, 387

Neisseria sicca, 387

Neisseria subflava, 387

Neisseria elongata, 387

Neisseria weaveri, 388

Bibliography, 389

OBJECTIVES

After reading and studying this chapter, you should be able to:

1. Describe the general characteristics of the genus *Neisseria*.
2. List the major pathogens within the genus *Neisseria*.
3. Describe the major virulence factors of the pathogenic *Neisseria* species and their function.
4. Compare gonorrhea in male and female patients, including potential complications of asymptomatic infections in women.
5. Discuss the pathology of ophthalmia neonatorum and how it can be prevented.
6. Determine the optimal specimen collection and transport methods for isolating *Neisseria gonorrhoeae*.

7. Compare the usefulness of direct Gram stain in the diagnosis of gonorrhea in men and women.
8. Describe nonselective and selective media for the isolation of *N. gonorrhoeae* and *Neisseria meningitidis*.
9. Summarize the culture requirements for the isolation of *N. gonorrhoeae* and *N. meningitidis*.
10. Discuss the tests used in identification of *N. gonorrhoeae* and *N. meningitidis* in cultures, distinguishing between presumptive tests and definitive methods.
11. Justify the use of nucleic acid amplification tests for the detection of *N. gonorrhoeae*.
12. Discuss modes of antimicrobial resistance in *N. gonorrhoeae* isolates and current recommendations for antimicrobial therapy.
13. Describe the pathogenesis of clinical infections caused by *N. meningitidis*.
14. Identify the high-risk groups for acquiring *N. meningitidis* infection.
15. Justify the use of the meningococcal vaccine in various populations.
16. Describe the specimens for the recovery of *N. meningitidis*.
17. Discuss the clinical significance of *Moraxella catarrhalis* in children and adults.
18. Summarize the laboratory features of *M. catarrhalis*, including specimens, media, colony morphology, and identification tests.
19. Characterize the commensal *Neisseria* spp., and differentiate it from the pathogenic *Neisseria* spp.

KEY TERMS

Capnophilic
Fitz-Hugh–Curtis syndrome
Gonorrhea
Lipooligosaccharide (LOS)
Ophthalmia neonatorum
Pelvic inflammatory disease

Penicillinase-producing *Neisseria gonorrhoeae* (PPNG)
Waterhouse-Friderichsen syndrome

Case in point

An 18-year-old sexually active college student on the women's gymnastics team visited student health services complaining of pain, redness, and swelling of both wrist joints and the left elbow. She gave a history of casual, unprotected sexual intercourse with "three or four" men during the past 4 months. She denied any vaginal discharge or abdominal pain but recalled having had a rash recently on both her arms. An aspirate from the left wrist was sent to the laboratory for culture and Gram stain. Direct Gram-stained smear of the aspirate showed the following: "Many polymorphonuclear white blood cells, rare intracellular and extracellular gram-negative diplococci seen." At 24 hours, many small, tan colonies were visible on chocolate (CHOC) agar, but there was no growth on sheep blood agar (SBA), the Centers for Disease Control and Prevention (CDC) anaerobic blood agar, or MacConkey agar. The organism was identified according to routine laboratory protocol.

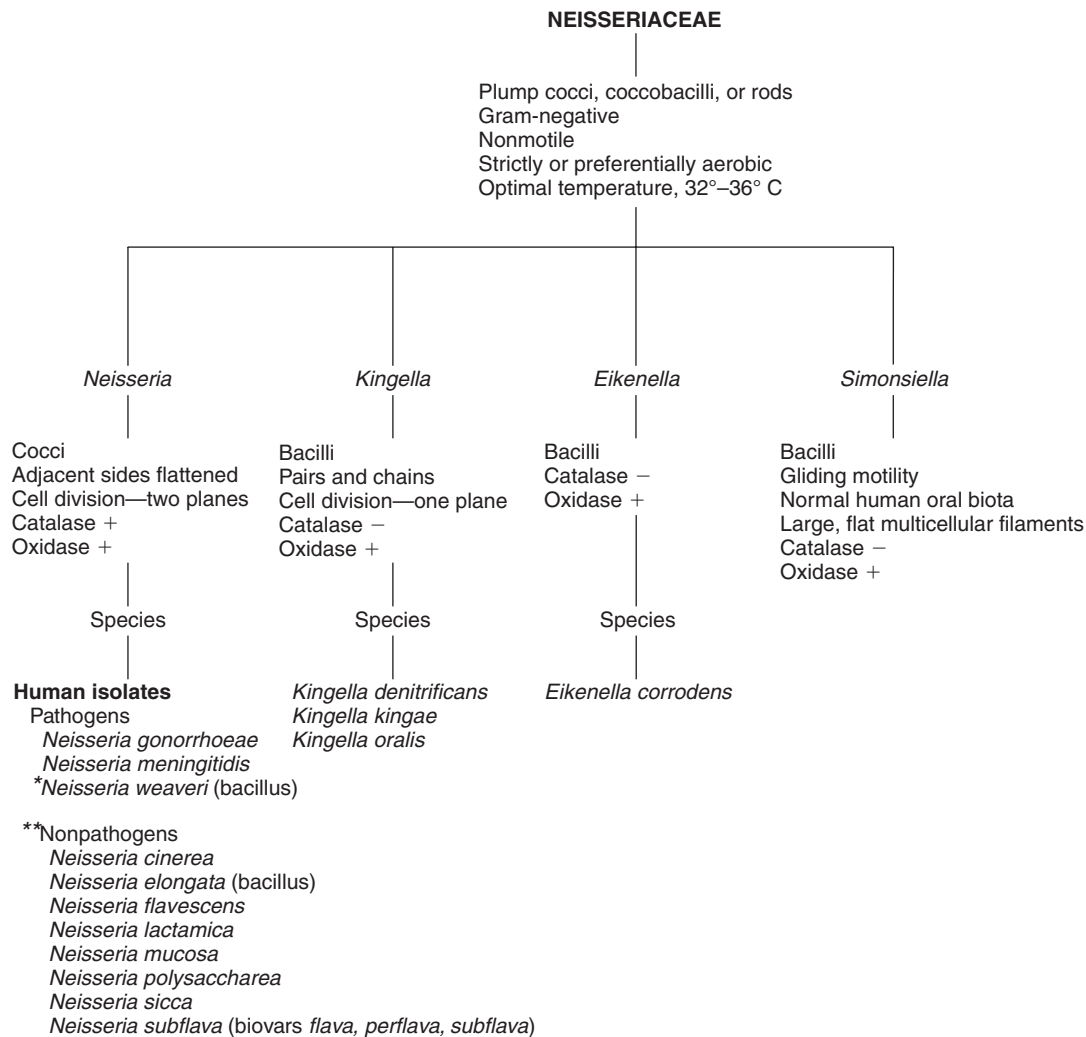
Issues to consider

After reading the patient's case history, consider:

- The identity of the organism causing the patient's infection

- Risk factors for acquisition of the organism
- Types and consequences of sequelae of untreated primary infection in female and male patients
- Importance of definitive identification of the microorganism

The family Neisseriaceae contains the genus *Neisseria* as well as *Kingella*, *Eikenella*, *Simonsiella*, *Allysiella*, and several other genera. *Moraxella catarrhalis* is in the family Moraxellaceae with other *Moraxella* spp., *Acinetobacter*, *Psychrobacter*, and several other genera. *M. catarrhalis* is included in this chapter because its cellular morphology resembles the morphology of *Neisseria*. Characteristics of the family Neisseriaceae and differential characteristics of these genera are shown in Fig. 17.1. Currently, the genus *Neisseria* contains approximately 40 species; about 17 species and biovars can be isolated from humans. This chapter discusses the *Neisseria* spp. and the morphologically and biochemically similar *M. catarrhalis*. The genera *Eikenella* and *Kingella* are discussed in Chapter 18.



*Normal oral biota of dogs, wounds from dog bites. **May cause opportunistic infections in human hosts.

Fig. 17.1 Characteristics of the family Neisseriaceae. *Moraxella catarrhalis*, although morphologically and biochemically similar to *Neisseria* spp., is not a member of the family Neisseriaceae; therefore it is not included here.

General characteristics

Most *Neisseria* spp. are aerobic, nonmotile, non-spore-forming, gram-negative diplococci. *Neisseria elongata*, *Neisseria weaveri*, and *Neisseria bacilliformis* are known exceptions, being rod shaped. All species are cytochrome oxidase and catalase positive except for *N. elongata* subsp. *nitroreducens* and *N. bacilliformis*, which are catalase negative. Many *Neisseria* spp. are capnophilic, requiring carbon dioxide (CO₂) for growth, and have optimal growth in a humid atmosphere. They can grow anaerobically if alternative electron acceptors (e.g., nitrites) are available. The natural habitats of *Neisseria* spp. are the mucous membranes of the respiratory and urogenital tracts of various animals. Table 17.1 shows the pathogenicity and host range of *Neisseria* spp. and *M. catarrhalis*.

Neisseria gonorrhoeae (often called gonococci) and *Neisseria meningitidis* (meningococci) are the primary human pathogens of the genus. *N. gonorrhoeae* is not considered part of the normal biota and is always pathogenic. *N. meningitidis* may be found as a commensal inhabitant of the upper respiratory tract of carriers, but it can also become an invasive pathogen. Pathogenic *Neisseria* spp. are fastidious organisms, requiring enriched media for optimal recovery, and are sensitive to unfavorable environmental conditions. Both *N. gonorrhoeae* and *N. meningitidis* require iron for growth. They compete with their human host by binding human transferrin to specific surface receptors. Their ability to bind transferrin may be a primary reason that they are strict human pathogens. *N. weaveri*, which is a commensal in the upper respiratory tract

of dogs, has been isolated from dog bites in humans. All other *Neisseria* spp. are considered opportunistic pathogens. These opportunists must be recognized and differentiated from *N. gonorrhoeae* and *N. meningitidis* in isolates from clinical specimens.

Pathogenic *Neisseria* species

Virulence factors

Pathogenic *Neisseria* spp. have several characteristics that contribute to their virulence, including the following:

- Receptors for human transferrin
- Capsule (*N. meningitidis*)
- Pili (fimbriae)
- Cell membrane proteins
- Lipooligosaccharide (LOS) or endotoxin; lipid A moiety and core LOS of lower molecular weight that differentiates it from the lipopolysaccharide found in most gram-negative bacilli and is loosely attached to the underlying peptidoglycan
- Immunoglobulin A (IgA) protease that cleaves IgA on mucosal surfaces

N. gonorrhoeae is divided into five morphologically distinct colony types. Types T1 to T5 are based on the presence or absence of pili, fine hairlike projections that are important in the initial attachment of the organism to host tissues. Pili also inhibit phagocytosis of the organism by neutrophils and aid in the exchange of genetic material between cells. Types T1 and T2, which possess pili, are virulent forms, whereas types T3 to T5 are devoid of pili and are avirulent. Piliated organisms usually predominate when first isolated from uncomplicated urogenital tract infections, but on subculture, pili are lost, and colony types T3 to T5 appear.

The cell outer membrane proteins I, II, and III and LOS not only serve as protective devices of the organism but are also important in antigenic variation. *N. meningitidis* possesses a polysaccharide capsule that is antiphagocytic and serves as an important virulence factor. Twelve serotypes of capsular antigens have been identified. The LOS endotoxin is a major in vivo virulence factor that mediates damage to body tissues and elicits an inflammatory response. During periods of rapid growth, the organism releases outer membrane fragments called blebs, which contain LOS. The major outer membrane porin protein (Por), or protein I, forms channels for nutrients to pass into and waste products to exit the cell. They are coded for by two genes: *porA* and *porB*. Both genes are expressed in *N. meningitidis*, but only *porB* is expressed in *N. gonorrhoeae*. *porB* is also protective against the host's inflammatory response and serum complement-mediated killing.

Protein II (Opa, for opacity) is a group of proteins that facilitate the adherence to phagocytic and epithelial cells. Protein III (reduction modified protein) blocks host serum bactericidal (IgG) action against the organism. A schematic diagram of the cellular structure of pathogenic *Neisseria* is shown in Fig. 17.2.

Table 17.1 Pathogenicity and host range for species of *Neisseria* and *Moraxella catarrhalis*

Species	Pathogenicity	Infected host
<i>N. gonorrhoeae</i>	Primary pathogen	Humans only
<i>N. meningitidis</i>	Primary pathogen	Humans only
<i>N. lactamica</i>	Opportunistic pathogen	Warm-blooded animals
<i>N. sicca</i>	Opportunistic pathogen	Warm-blooded animals
<i>N. subflava</i>	Opportunistic pathogen	Warm-blooded animals
<i>N. mucosa</i>	Opportunistic pathogen	Warm-blooded animals
<i>N. flavescens</i>	Opportunistic pathogen	Warm-blooded animals
<i>N. cinerea</i>	Opportunistic pathogen	Warm-blooded animals
<i>N. polysaccharea</i>	Opportunistic pathogen	Warm-blooded animals
<i>N. elongata</i>	Opportunistic pathogen	Warm-blooded animals
<i>N. bacilliformis</i>	Opportunistic pathogen	Warm-blooded animals
<i>M. catarrhalis</i>	Opportunistic pathogen	Humans only

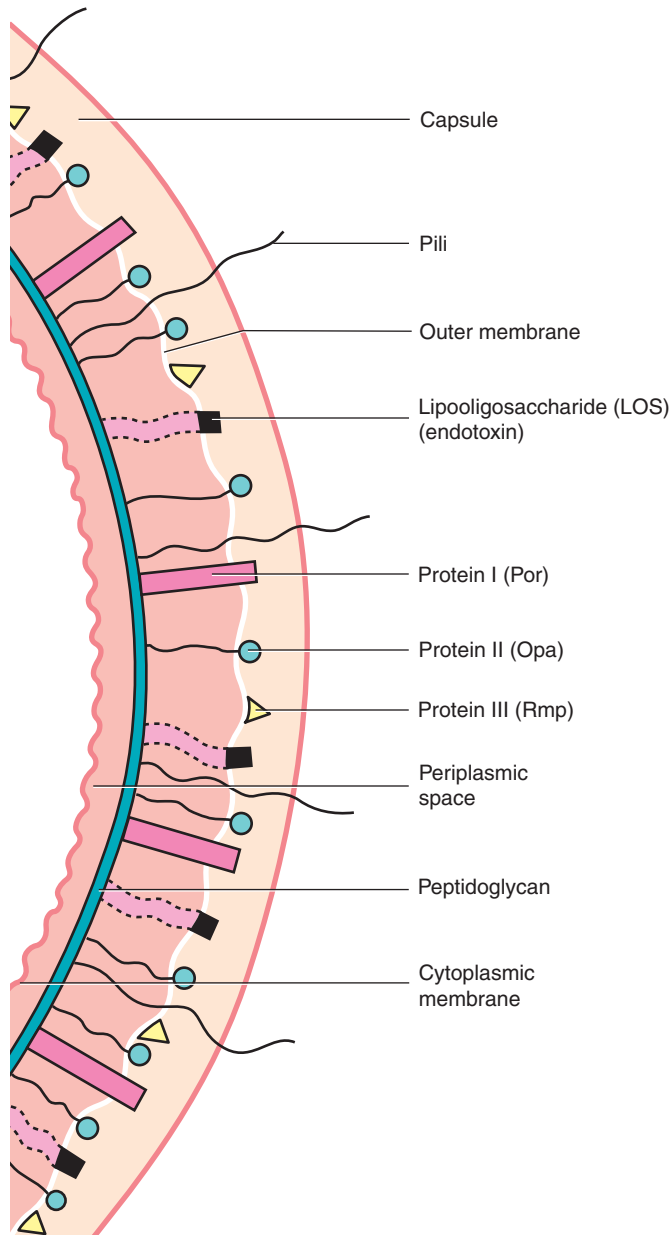


Fig. 17.2 Cellular structure of the cell wall of pathogenic *Neisseria* spp.

Neisseria gonorrhoeae

Humans are the only natural host for *N. gonorrhoeae*, the agent of the sexually transmitted disease **gonorrhea**. Gonorrhea is an acute pyogenic infection of nonciliated columnar and transitional epithelium; infection can be established at any site where these cells are found. Gonococcal infections are mostly acquired through sexual contact and occur primarily in the urethra, endocervix, anal canal, pharynx, and conjunctiva. Disseminated infections from the primary site may also occur, as seen in the Case in Point.

The first use of the term *gonorrhea*, meaning a “flow of seed,” was in the second century when the urethral discharge was mistaken for semen. For centuries thereafter, the diseases syphilis and gonorrhea were confused because the two were often present together in the same individual. In 1530, it was thought that gonorrhea was an early symptom of syphilis.

The issue was further confused in 1767 because of a classic blunder by a physician who inoculated himself intentionally with pus from a patient with symptoms of gonorrhea. The pus gave the physician syphilis instead. Gonorrhea was also called *the clap*, from the French word *clapoir* meaning “brothel.”

Epidemiology

N. gonorrhoeae infections are most commonly transmitted through **sexual contact**. The primary reservoir is the **asymptomatic carrier**. In the United States, gonorrhea is a reportable disease nationally; all culture-confirmed cases must be reported to state health laboratories. Gonorrhea is second to *Chlamydia trachomatis* in the number of confirmed sexually transmitted bacterial infections in the United States. However, human papillomavirus, a nonreportable infectious agent, is estimated to be the most common cause of sexually transmitted diseases in the United States. There were 677,769 cases of gonorrhea reported in 2020, an increase of 45% from 2016. Actual numbers of infected individuals are probably much higher than reported because of a large reservoir of asymptomatic carriers and other unreported cases. Sexually active teens and young adults have the highest rates of infection. Most cases in both men and women are seen between **20 and 24 years of age**. In the United States, the highest rates are seen in high-density, urban areas, where individuals are more likely to have multiple partners and unprotected sexual intercourse.

Clinical infections

Gonorrhea has a short incubation period of approximately 2 to 7 days. In men, acute urethritis, usually resulting in purulent discharge and dysuria (painful urination), are the most common manifestations. Asymptomatic gonococcal infection in men is uncommon; up to 10% of cases are asymptomatic. *N. gonorrhoeae* strains with a nutritional requirement for **arginine, hypoxanthine, and uracil (AHU)** are often isolated from asymptomatic men. Complications in male patients include ascending infections, such as prostatitis and epididymitis.

The endocervix is the most common site of infection in women. Symptoms of infection, when present, include dysuria, cervical discharge, and lower abdominal pain. However, 50% of cases in women may be asymptomatic, leading to complications, such as **pelvic inflammatory disease**, which may cause sterility, ectopic pregnancy, or perihepatitis (**Fitz-Hugh–Curtis syndrome**).

Blood borne dissemination of *N. gonorrhoeae* occurs in less than 1% of all infections, resulting in purulent arthritis and rarely septicemia. Fever and a rash on the extremities can also be present. Most disseminated gonococcal infections are attributed to the AHU strains and occur in women. Because *N. gonorrhoeae* is inhibited by **sodium polyanethol sulfonate (SPS)**, the anticoagulant in blood culture media, it might not be recovered from blood cultures. **Gelatin can be added to the media to neutralize the effects of SPS.**

Other conditions associated with *N. gonorrhoeae* include anorectal and oropharyngeal infections. Infections in these sites are more common in men who have sex with men but can also occur in women. Most of these infections are asymptomatic or have nonspecific symptoms. **Pharyngitis is the**

chief complaint in symptomatic oropharyngeal infections, whereas discharge, rectal pain, or bloody stools may be seen in rectal gonorrhea. Approximately 30% to 60% of women with genital gonorrhea have concurrent rectal infection.

Newborns can acquire *ophthalmia neonatorum*, a gonococcal eye infection, during vaginal delivery through an infected birth canal. This condition, which can result in blindness if not treated immediately, is rare in the United States because application of antimicrobial eye drops, generally erythromycin, at birth to every infant is legally required. Ocular infections can occur in adults because of self-inoculation of the eye with infected genital secretions or, rarely, as a result of a laboratory accident.

Case check 17.1

The most common method for acquisition of *N. gonorrhoeae* infection is sexual contact. The endocervix is the most common site of infection in women; however, 50% of women are asymptomatic. The Case in Point demonstrates the risk factors for acquiring gonorrhea and, more importantly, the consequences of the sequelae of untreated infections in women. The patient had unprotected sexual intercourse with multiple partners. Because she did not have genital symptoms of gonorrhea, the organism went undetected, and *N. gonorrhoeae* infection was disseminated, resulting in septic arthritis. Septic arthritis in a patient of this age is rare, and when it occurs, the laboratory should consider *N. gonorrhoeae* as a potential causative agent.

Laboratory diagnosis

Specimen collection and transport

Specimens collected for the recovery of *N. gonorrhoeae* may be taken from the **genital tract or other sites, such as the rectum, pharynx, and joint fluid**. The laboratory should be notified when cultures for *N. gonorrhoeae* from sites other than the genital tract are requested because normal laboratory protocols for such specimens would likely not recover the organism.

The specimen of choice for genital infections in men is the urethra and in women is the endocervix. In men, purulent discharge can be collected directly onto a swab for culture. When no apparent discharge is present, the swab is inserted up to 2 cm into the anterior urethra and slowly rotated to collect material. Swabs for rectal culture should be inserted 4 to 5 cm into the anal canal. Disinfectants should be avoided in preparing the patient for collection of the specimen.

Calcium alginate and cotton swabs are inhibitory to *N. gonorrhoeae*, so Dacron or rayon swabs are preferred. Because *N. gonorrhoeae* is extremely susceptible to drying and temperature changes, direct plating of the specimen to gonococcal-selective media gives optimal results. Several commercial transport systems, such as James E. Martin Biological Environmental Chamber (JEMBEC) plates (Becton Dickinson Diagnostics, Sparks, MD) (Fig. 17.3), Gono-Pak (Becton Dickinson Diagnostics), and Transgrow (Hardy Diagnostics, Santa Maria, CA), allow direct plating in the clinical setting. These products contain selective media and a CO₂ atmosphere to provide optimal conditions until the specimen reaches the laboratory. The swab containing the specimen should be rolled in a Z pattern on the medium. On receipt in the laboratory, the media can be cross-streaked with a loop to



Fig. 17.3 Growth of *Neisseria gonorrhoeae* after 24 hours on a James E. Martin Biological Environmental Chamber (JEMBEC) plate inoculated in a characteristic Z pattern.

facilitate growth of isolated colonies. When direct plating is not possible, inoculated swabs should be placed in a transport system, such as Amies medium with charcoal; transported to the laboratory promptly; and plated within 6 hours of collection. **Do not refrigerate specimens for the recovery of *N. gonorrhoeae*.**

Direct microscopic examination

Smears for direct Gram stain should be prepared from urogenital specimens. Gram stain is not recommended for pharyngeal specimens because commensal *Neisseria* spp. can be present. The demonstration of gram-negative intracellular diplococci from the urethral discharge of a symptomatic male correlates at a rate of 89% with culture and is evidence of gonococcal infection. The gonococci are in pairs with adjacent sides flattened, giving them a kidney shape (Fig. 17.4A). Many times, avirulent forms (lacking pili) of the organism are seen as extracellular, gram-negative diplococci in the direct smear of the clinical specimen.

Because women have vaginal commensal microbiota that resembles gonococci, direct Gram stain correlates in only 50% to 70% of cases with culture. Direct Gram stain may be helpful in a symptomatic woman with discharge, but culture is necessary for confirmation. Gram stain with more than five polymorphonuclear neutrophils per high-power field but no bacteria (see Fig. 17.4B) suggests nongonococcal urethritis with other organisms, such as *C. trachomatis* or *Ureaplasma urealyticum*.

Culture

The medium of choice for cultivation of *N. gonorrhoeae* is CHOC agar. Typically, *N. gonorrhoeae* is described as an organism that does not grow on SBA. In recent years, however, some strains will grow, although slowly if incubated in an atmosphere of increased CO₂. It is not certain if this is caused by a change in the strains or in the increased enrichment of commercially prepared SBA. Because CHOC agar supports the growth of many organisms found as commensals in specimens collected for the recovery of gonococci, a selective medium is necessary. Commonly used selective media are described in Table 17.2. **All of these media contain vancomycin and colistin to inhibit gram-positive and**

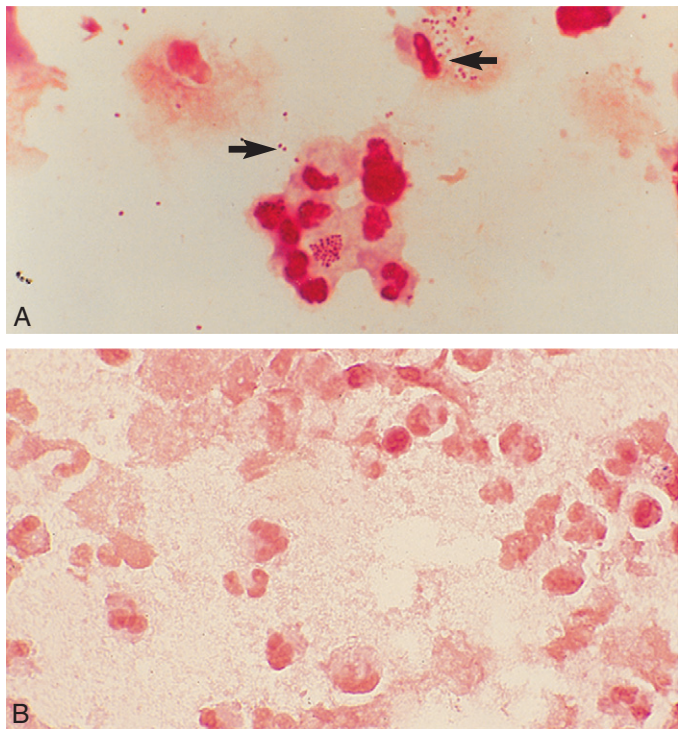


Fig. 17.4 **A**, Direct Gram-stained smear of male urethral discharge showing intracellular and extracellular gram-negative diplococci (arrows), which is diagnostic of *Neisseria gonorrhoeae* (×1000). **B**, Direct smear with more than five polymorphonuclear neutrophils per high-power field but no bacteria may suggest nongonococcal urethritis (×1000).

gram-negative bacteria, along with an antifungal agent to suppress the growth of yeast. Trimethoprim is used in most of these media to prevent growth of the swarming of *Proteus* spp. To recover vancomycin-sensitive strains of gonococci, many laboratories include a CHOC agar plate as a primary plating medium. Several other bacteria grow on selective gonococcal media, including *Acinetobacter* spp., *Capnocytophaga* spp., and *Kingella denitrificans*. These isolates can be differentiated from *N. gonorrhoeae* by the oxidase and catalase tests. All specimens received in the laboratory for recovery of *Neisseria* spp. should be held at room temperature and plated as soon as possible. Because *Neisseria* spp. are susceptible to cold temperatures, media should be warmed to room temperature before inoculation.

Incubation

Inoculated plates should be incubated at 35°C in a 3% to 5% CO₂ atmosphere. Incubation is accomplished by using a CO₂ incubator, a CO₂ generating pouch, or a candle extinction jar. Plates are put into a large glass jar, where a lit candle is added, and the lid is closed. The candle burns until the oxygen concentration decreases to the point that the flame is extinguished. Candle jars obtain a CO₂ of about 3%. Scented or colored candles may be inhibitory to the gonococci, so only white, unscented wax candles are used in the candle extinction jar. Sufficient humidity is provided by the moisture evaporating from the media in a closed jar. Most microbiology laboratories use CO₂ incubators that are automatically humidified, or a pan of water can be placed in the bottom. Cultures are examined daily for growth and held for 72 hours.

Presumptive identification

Colony morphology

Colonies of *N. gonorrhoeae* on CHOC agar or selective agars are small, gray to tan in color, translucent, and raised after 24 to 48 hours of incubation (Fig. 17.5). As noted previously, five colony types of *N. gonorrhoeae* have been described: T1

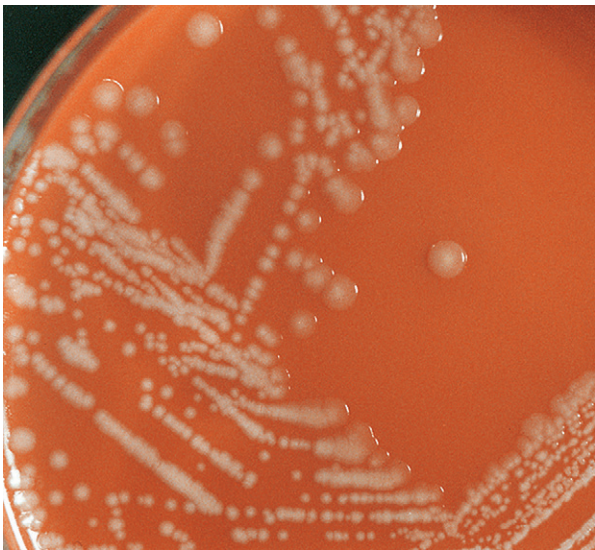


Fig. 17.5 *Neisseria gonorrhoeae* colony morphology after 24 hours of growth on Modified Thayer-Martin (MTM) agar.

Table 17.2 Selective media for isolation of <i>Neisseria gonorrhoeae</i> and <i>Neisseria meningitidis</i>		
Selective medium	Inhibitory agents	Suppressed organisms
Thayer-Martin	Vancomycin	Gram-positive
	Colistin	Gram-negative
	Nystatin	Yeast
Modified Thayer-Martin (MTM)	Vancomycin	Gram-positive
	Colistin	Gram-negative
	Nystatin	Yeast
	Trimethoprim	Swarming <i>Proteus</i> spp.
Martin-Lewis	Vancomycin	Gram-positive
	Colistin	Gram-negative
	Anisomycin	Yeast
	Trimethoprim	Swarming <i>Proteus</i> spp.
New York City	Vancomycin	Gram-positive
	Colistin	Gram-negative
	Amphotericin B	Yeast
	Trimethoprim	Swarming <i>Proteus</i> spp.
GC-LECT	Vancomycin	Gram-positive
	Lincomycin	Gram-positive
	Colistin	Gram-negative
	Amphotericin B	Yeast
	Trimethoprim	Swarming <i>Proteus</i> spp.

and T2 have pili and are considered virulent; these colonies are smaller and raised, and they appear bright in reflected light. Types T3 to T5 do not have pili and usually grow as larger, flatter colonies. AHU strains produce smaller colonies and grow more slowly; they are often more difficult to identify with biochemical methods. The gonococci can produce autolytic enzymes that may make the isolate nonviable on prolonged incubation. A fresh subculture should be used for identification tests.

Microscopic morphology

Gram stain must be performed on all suspected *N. gonorrhoeae* isolates to verify the appearance of gram-negative diplococci. Some gram-negative rods, such as *Kingella* and *Acinetobacter* spp., are occasionally able to grow on gonococcal selective media. To differentiate these from gram-negative diplococci, the organism can be streaked to a plate with a 10-unit penicillin disk added (Fig. 17.6). After growth, colonies at the edge of the zone of inhibition are taken and Gram-stained to visualize the morphology.

Oxidase test

The oxidase test must be done on all suspected isolates of *N. gonorrhoeae*. In the filter paper method, oxidase reagent (1% dimethyl-*p*-phenylenediamine dihydrochloride or tetramethyl-*p*-phenylenediamine dihydrochloride) is placed on filter paper, and a colony from the plate is rubbed onto the reagent with an applicator stick or a non-nichrome loop. In a positive reaction on a fresh isolate, a purple color should develop within 10 seconds (Fig. 17.7A). Alternatively, the oxidase reagent may be dropped directly onto a colony. In a positive reaction, the colony turns deep purple to black (see Fig. 17.7B). Although the direct plate method is convenient, the reagent renders organisms nonviable, so if subculture is needed, it must be done before adding the reagent. Commercial oxidase strips are also available.

Definitive identification

Other oxidase-positive, gram-negative diplococci, such as *Neisseria cinerea*, *N. meningitidis*, and *M. catarrhalis*, can grow on selective media from sites where *N. gonorrhoeae* is

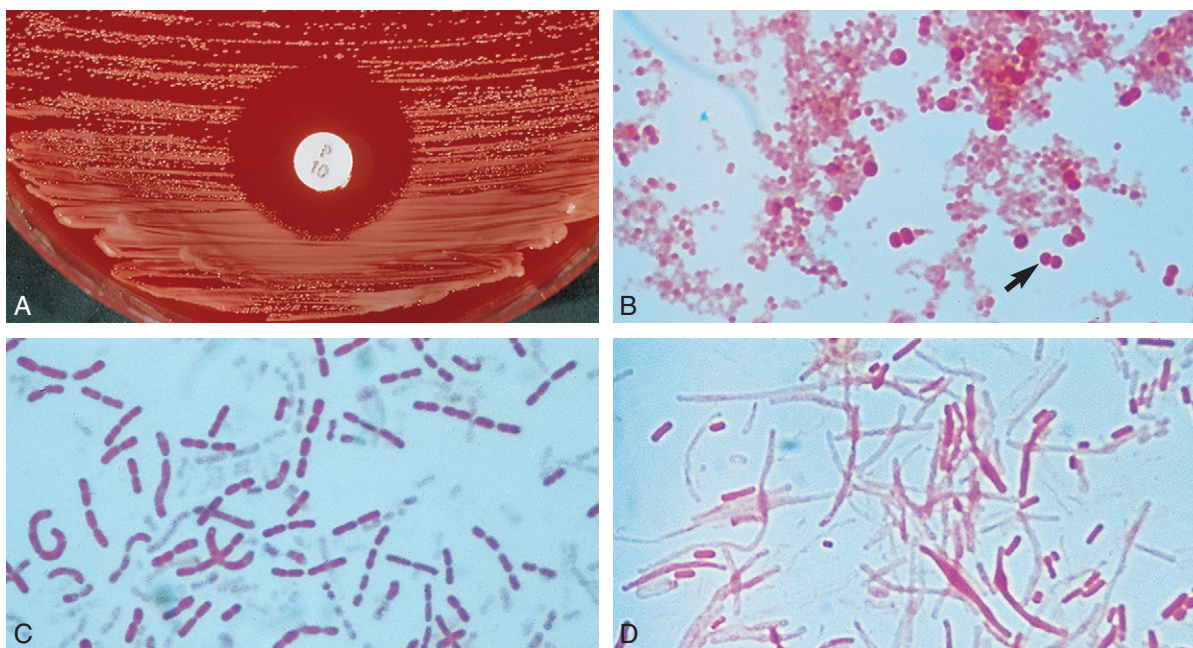


Fig. 17.6 A, To differentiate some gram-negative rods from gram-negative diplococci, the organism can be streaked to a plate, and a 10-unit penicillin disk can be added. After growth, colonies at the edge of the zone of inhibition are Gram-stained to visualize microscopic morphology. B, The microscopic morphology of *Neisseria gonorrhoeae* remains gram-negative diplococci (arrow) using the 10-unit penicillin disk ($\times 1000$). C, The gram-negative rod microscopic morphology of *Kingella* spp. after the penicillin disk test ($\times 1000$). D, The elongated gram-negative rod microscopic morphology of *Acinetobacter* spp. after the penicillin disk test ($\times 1000$).

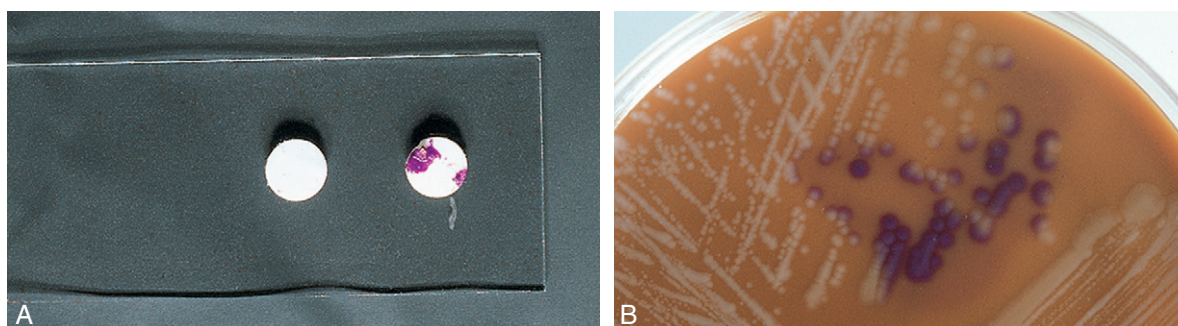


Fig. 17.7 A, The oxidase disk test. The negative control is on the left, and the positive reaction (purple) is on the right. B, Example of a positive oxidase reaction when the reagent is dropped directly on the colony.

expected. These organisms would be incorrectly reported as *N. gonorrhoeae* if no further identification was performed. Many different methods are available for the identification of *Neisseria* and *Moraxella* spp. or for confirmation only of *N. gonorrhoeae* isolates. See Table 17.3 for a description of testing methods. The selection of a particular method depends on the demographic profile of patients, sensitivity and specificity of the method with low-prevalence or high-prevalence groups, cost of materials to perform procedure, time, and number of tests performed.

Carbohydrate utilization

The traditional method for the identification of *Neisseria* spp. was carbohydrate utilization in cystine trypticase agar (CTA) containing 1% of the individual carbohydrate and phenol red as a pH indicator. If the organism uses the particular carbohydrate, acid, characterized by a yellow color, is produced in 24 to 72 hours. *N. gonorrhoeae* is positive for glucose only. Many problems are associated with this method, however, and it has been replaced by rapid, more accurate tests (see Table 17.3).

The rapid carbohydrate degradation tests require pure cultures but can be read in 2 to 4 hours, rather than the 24 to 72 hours needed for the CTA carbohydrate test. These rapid tests also detect acid production from various carbohydrates, but they are based on the presence of preformed enzymes

for carbohydrate utilization rather than on bacterial growth. Problems noted with these methods include:

- Weak acid production from glucose by certain strains of *N. gonorrhoeae*
- Misidentification of sucrose-negative strains of *Neisseria subflava* as *N. meningitidis*
- Strains of *N. cinerea* that give positive glucose reactions

Chromogenic substrates

Chromogenic substrate methods, such as the Gonocheck II (EY Laboratories, San Mateo, CA), detect enzymes that hydrolyze colorless substrates and produce colored end products. Only strains that are isolated on selective media should be tested. The advantage of these tests is the identification of strains of *Neisseria* spp. with aberrant carbohydrate utilization. Problems noted with these tests include misidentification of nonpathogenic species, such as *N. cinerea*, *Neisseria sicca*, *N. subflava*, and *N. mucosa*, as *N. gonorrhoeae* or *N. meningitidis*.

Multitest methods

The multitest conventional-chromogenic enzyme methods combine enzyme substrate tests with other biochemical tests and allow for identification of strains isolated on selective or nonselective media. These tests can also identify

Table 17.3 Selected culture-based test methods for identification of *Neisseria* and related species

Method type and name	Manufacturer ^a	Principle	Comments
Conventional			
Cystine trypticase agar with 1% carbohydrates	Various	Acid production from carbohydrate utilization	Requires pure culture; must be incubated 24–72 hours; no longer recommended
Chromogenic substrate			
Gonocheck II	EY Laboratories ¹	Detects enzyme production	Identifies isolates only from selective media
BactiCard <i>Neisseria</i>	Thermo Fisher Scientific ²		
Coagglutination			
Phadebact Monoclonal GC	MKL Diagnostic AB ³	Monoclonal antibodies used to detect <i>N. gonorrhoeae</i>	
Modified conventional/chromogenic enzyme			
BBL Crystal <i>Neisseria</i> / <i>Haemophilus</i> ID Kit	BD Diagnostic Systems ⁴	Multitest systems—combine enzyme substrate with other biochemical tests	Identifies isolates from selective or nonselective media; identifies <i>Neisseria</i> spp. and <i>Haemophilus</i> spp.
CarboFerm <i>Neisseria</i> Test	Hardy Diagnostics ⁵		Identifies <i>Neisseria</i> spp. and <i>M. catarrhalis</i>
Vitek <i>Neisseria</i> - <i>Haemophilus</i> Identification	bioMérieux ⁶		Identifies <i>Neisseria</i> spp., <i>Haemophilus</i> spp., <i>M. catarrhalis</i> , and other fastidious organisms
RapID NH System	Thermo Fisher Scientific ²		Identifies <i>Neisseria</i> spp., <i>Haemophilus</i> spp., <i>M. catarrhalis</i> , and other fastidious organisms
Microscan HNID Panel	Beckman Coulter ⁷		Identifies <i>Neisseria</i> spp., <i>Haemophilus</i> spp., and <i>M. catarrhalis</i>
API NH	bioMérieux		Identifies <i>Neisseria</i> spp., <i>Haemophilus</i> spp., and <i>M. catarrhalis</i>

^aLocations for manufacturers are as follows: ¹San Mateo, CA; ²Waltham, MA; ³Sollentuna, Sweden; ⁴Franklin Lakes, NJ; ⁵Santa Maria, CA; ⁶Durham, NC; and ⁷Brea, CA.

other genera, such as *Haemophilus*. Characteristics of clinically significant species of *Neisseria*, *Moraxella*, and *Kingella* are listed in Table 17.4. Key differentiating reactions of the major pathogens include use of glucose only by *N. gonorrhoeae*, whereas *N. meningitidis* uses glucose and maltose. *M. catarrhalis* is asaccharolytic but, in contrast with *Neisseria* spp., is deoxyribonuclease (DNase) and butyrate esterase positive.

Immunologic assays

Immunologic methods employ monoclonal antibodies against gonococcal protein I for the identification of *N. gonorrhoeae*. These methods do not require pure or viable organisms and can be done from the primary plates. However, they are not approved for use on clinical specimens. Immunologic methods include coagglutination and fluorescent antibody testing. Coagglutination tests use monoclonal antibodies directed against *N. gonorrhoeae* attached to killed *Staphylococcus aureus* cells; agglutination is a positive reaction. Sensitivity of the assays is generally high, but false-positive results because of cross-reaction with *N. cinerea*, *N. lactamica*, and *N. meningitidis* have been reported.

Matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry

Matrix-assisted laser desorption/ionization–time-of-flight (MALDI-TOF) mass spectrometry is an emerging technology that identifies infectious pathogens by defining unique protein signatures of the organism (see Chapter 11). An isolated colony is ionized, resulting in vaporization of the proteins. The proteins are separated based on size and charge in an electric field, resulting in a unique spectral signature. The use of MALDI-TOF mass spectrometry has improved microbiology testing by allowing for identification of a pathogen in minutes. However, identification of closely related species of organisms can be challenging. Studies indicate that the identification of *N. gonorrhoeae* by MALDI-TOF mass spectrometry is highly accurate.

Nucleic acid amplification tests

Nucleic acid amplification tests (NAATs) are the preferred assays for the detection of *N. gonorrhoeae* in clinical specimens because of increased sensitivity, specificity, and ability to test with a noninvasive urine specimen. NAATs amplify a specific nucleic acid sequence (e.g., polymerase chain reaction) before

Table 17.4 Characteristics of significant species of *Neisseria*, *Moraxella*, and *Kingella*

Characteristic	<i>N. gonorrhoeae</i>	<i>N. meningitidis</i>	<i>N. lactamica</i>	<i>N. cinerea</i>	<i>N. sicca</i>	<i>N. flavescens</i>	<i>Moraxella</i>	<i>Kingella</i>
Pigment on nutrient agar	–	–	–	–	+	+	–	–
Catalase (3% hydrogen peroxide [H ₂ O ₂])	+	+	+	+	+	+	+	–
Superoxol (30% H ₂ O ₂)	+	–	–	–	–	–	–	–
Growth on								
MTM, ML, NYC	+	+	+	d	–	–	d	+
Nutrient medium at 35°C	–	–	+	+	+	+	+	–
Acid production from								
Glucose	+	+	+	+ ^a	+	–	–	+
Maltose	–	+	+	–	+	–	–	–
Sucrose	–	–	–	–	+	–	–	–
Lactose	–	–	+	–	–	–	–	–
Fructose	–	–	–	–	+	–	–	–
DNase	–	–	–	–	–	–	+	–
Reduction of								
Nitrite (NO ₃)	–	–	–	–	–	–	+	+
Nitrogen dioxide (NO ₂)	–	d	d	+	+	+	+	–
Tributyrin hydrolysis	–	–	–	–	–	–	+	NT
Enzymes produced								
β-D-Galactosidase	–	–	+	–	–	NT	–	–
γ-Glutamyl aminopeptidase	–	+	–	–	–	NT	–	–
Hydroxyprolylaminopeptidase	+	–	–	+	NT	+	–	+

+, Most strains (>90%) positive; –, most strains (>90%) negative; d, some strains positive, some negative; ML, Martin-Lewis agar; MTM, modified Thayer-Martin agar; NT, not tested; NYC, New York City medium.

^aOccasional strains may give weak glucose reactions in some rapid carbohydrate tests.

detecting the target sequence with a probe. Other methods rely on an amplified signal after a specific probe binds to the target sequence. NAATs are extremely sensitive and do not require viable organisms. Because urine can be used in these procedures, self-collection of the specimen is possible, and pelvic examination or intraurethral swabs are not required. First-morning voided urine from males is equivalent to or better than urethral swabs. In women, vaginal swabs have been demonstrated to be as sensitive as cervical swabs. First-morning voided urine samples from women can be used for screening, but they have been shown to detect up to 10% fewer cases compared with vaginal and cervical swabs. NAATs have the additional advantage of being less sensitive to transport and storage conditions compared with culture. NAATs can also detect both *N. gonorrhoeae* and *C. trachomatis* in the same specimen (Table 17.5).

In some situations, a NAAT is not recommended. In cases of child sexual assault involving boys and in extragenital infections (i.e., rectal, oropharyngeal) in prepubescent girls, culture is recommended because data are insufficient to recommend nonculture methods. NAATs can be used for testing vaginal or urine specimens from young girls, but culture is the preferred method for testing urethral specimens from boys or extragenital specimens from boys and girls. Culture is the only method to monitor the effectiveness of treatment because NAATs are not approved for use as a test of cure. Culture for *N. gonorrhoeae* is necessary to monitor antimicrobial resistance.

Antimicrobial resistance

In the United States, almost all strains of *N. gonorrhoeae* were previously susceptible to penicillin. In 1976, the first plasmid-mediated **penicillinase-producing *Neisseria gonorrhoeae* (PPNG)** strains were isolated, largely imported from Southeast Asia or Africa. By 1980, more than one half of the reported PPNG cases were of domestic origin. In addition to plasmid-mediated penicillin resistance, in which the organism acquires a plasmid with genes for β -lactamase production, *N. gonorrhoeae* can exhibit **chromosome-mediated penicillin resistance (PenR)**, which was initially noted in the United States in 1983. These isolates are β -lactamase negative. Resistance in these strains results from a combination of mutations at several chromosomal loci, resulting in altered penicillin-binding proteins.

Table 17.5 FDA-approved nucleic acid amplification test methods for identification of *Neisseria gonorrhoeae*

Name	Manufacturer ^a	Principle
Cobas 4800 CT/NG	Roche Diagnostics ¹	PCR amplification of target gene
BD Max CT/GC/TV	BD Diagnostic Systems ²	Strand displacement amplification
APTIMA GC	Hologic ³	Target-amplification
APTIMA Combo 2	Hologic	Target-amplification
Abbott RealTime CT/NG	Abbott Molecular ⁴	PCR amplification
Cepheid CT/NG Xpert	Cepheid ⁵	Real-time PCR

FDA, U.S. Food and Drug Administration; PCR, polymerase chain reaction.

From Elias, J., et al. (2019). *Neisseria*. In K. C. Carroll, et al. (Eds.), *Manual of clinical microbiology* (12th ed., p. 644). Washington, DC: ASM Press.

^aLocations for manufacturers are as follows: ¹Indianapolis, IN; ²Sparks, MD;

³Marlborough, MA; ⁴Des Plaines, IL.; ⁵Sunnyvale, CA.

In addition to penicillin resistance, *N. gonorrhoeae* exhibits plasmid-mediated resistance and **chromosome-mediated resistance to tetracycline, spectinomycin, and the fluoroquinolones (e.g., ciprofloxacin)**. Spectinomycin resistance, first reported in the United States in 1981, is caused by a chromosomal mutation resulting in high-level resistance to the antimicrobial agent.

Because of concerns over the development of antimicrobial resistance by *N. gonorrhoeae*, the CDC developed the Gonococcal Isolate Surveillance Project (GISP) in 1986. This sentinel surveillance system monitors gonococcal antimicrobial susceptibility to provide treatment recommendations and to recognize treatment failures before they become a public health problem.

During the 1990s and 2000s, fluoroquinolones were recommended for treatment of gonorrhea because they were safe, cost-effective, and available in oral forms. Fluoroquinolone resistance was recognized by GISP monitoring, and by 2007, the CDC no longer recommended the use of fluoroquinolones for gonorrhea. Cephalosporins are currently the only antimicrobials remaining that are recommended for treatment; however, isolates with high-level cefixime and ceftriaxone minimal inhibitory concentrations have been identified.

Treatment

The 2020 Treatment Guidelines for Gonococcal Infection recommend a single 500mg intramuscular dose of ceftriaxone for uncomplicated gonorrhea. If chlamydial infection cannot be excluded, oral doxycycline (100mg twice daily for 7 days) should also be administered for co-infection with *C. trachomatis*.

Monitoring for the emergence of ceftriaxone resistance is imperative to ensure continued efficacy of the recommended treatment protocol. Surveillance and the reporting of treatment failures by health care providers are essential to ensuring therapeutic success.

Case check 17.2

The organism causing the infection in the Case in Point is *N. gonorrhoeae*. Direct Gram stain of the wrist aspirate revealed the presence of this bacterium. *N. meningitidis* could also result in this clinical picture; therefore laboratory testing is imperative. Culture of the fluid provided a clue to the identity when growth occurred on CHOC agar with no growth on SBA. *N. gonorrhoeae* is one of the few pathogens (*Haemophilus influenzae* is another) that does not usually grow on SBA. Presumptive testing included Gram stain of the colonies and an oxidase test. Definitive testing with a commercial coagglutination test kit was performed. The laboratory tested for β -lactamase with the Cefinase test. Some laboratories continue to perform β -lactamase testing on gonococcal isolates, although this is no longer necessary because penicillin has not been considered a therapeutic option for several years.

Neisseria meningitidis

Similar to *N. gonorrhoeae*, *N. meningitidis* is also found only in humans. However, *N. meningitidis* can be found as a commensal as well as an invasive pathogen. It is an important etiologic agent of endemic and epidemic meningitis and

meningococcemia and rarely pneumonia, purulent arthritis, or endophthalmitis. *N. meningitidis* has also been recovered from urogenital and rectal sites as a result of oral-genital contact. Meningococcal carriage, usually involving non-encapsulated strains, may cause an increase in protective antibodies against the pathogenic strains.

Epidemiology

N. meningitidis can be found on the mucosal surfaces of the nasopharynx and oropharynx in 30% of the population. The organism is transmitted by close contact with respiratory droplet secretions from a carrier to a new host. Only a few newly colonized hosts develop meningococcal disease, with the highest incidence being found in infants and adolescents.

Increased risk of invasive disease occurs in individuals with asplenia or individuals with complement deficiencies. Smoking, exposure to smoke, or concurrent upper respiratory tract viral infection can also lead to serious disease. Crowded living conditions can facilitate the spread of meningococcus because individuals from different areas can be colonized by different strains. Military recruits and resident college students have an increased risk of exposure because of living in close quarters. In one study of university students, nasopharyngeal carriage increased significantly during the first few weeks of the academic year.

Of the 12 meningococcal encapsulated serogroups, A, B, C, Y, and W-135 account for most cases of disease in the world. Invasive meningococcal disease is uncommon in developed nations. Serogroups B, C, and Y are most common in the United States and Europe. Meningococcal disease in the United States has been declining since the 1990s. In 2019, 375 cases were reported to the CDC—an incidence rate of 0.11/100,000 population. An area of sub-Saharan Africa referred to as the “meningitis belt” frequently experiences epidemics during the hot and dry season. The infection rates are approximately 1000 per 100,000 in this area with a population of 300 million, with serogroup A historically accounting for most of the cases. In 2010, a mass serogroup A vaccine campaign was started in the meningitis belt countries, and epidemics caused by serogroup A have been eliminated. Recent outbreaks in this area have been primarily caused by serogroups C and W.

Clinical infections

After exposure to meningococcus, the incubation period ranges from 1 to 10 days. The bacteria adhere to the nasopharyngeal mucosa, leading to colonization. In most individuals, colonization of the mucosa results in a subclinical infection or mild symptoms. In a few hosts, the bacteria gain access to the bloodstream and potentially the central nervous system, resulting in meningitis, sepsis, or both.

When *N. meningitidis* enters the bloodstream, two main diseases can occur: fulminant meningococcemia or meningitis. Meningococcemia, or sepsis, may occur with or without meningitis and carries a 25% mortality rate, even if treated. Purpura (hemorrhaging of blood into skin and mucous membranes, producing bruises) with petechial skin rash (pinpoint red spots caused by hemorrhage) (Fig. 17.8A), tachycardia, and hypotension can develop during bacteremia, and thrombosis is common. In some cases, the disease becomes

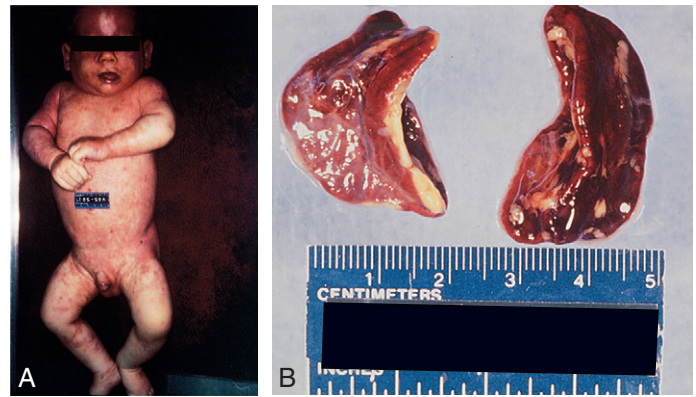


Fig. 17.8 A, Petechial skin rash associated with meningococcemia in an infant. B, Hemorrhage (dark red areas) in the adrenal glands in Waterhouse-Friderichsen syndrome.

fulminant and spreads rapidly, causing disseminated intravascular coagulation, septic shock, or hemorrhage in the adrenal glands (Waterhouse-Friderichsen syndrome) (see Fig. 17.8B). Death may occur in 12 to 48 hours from onset. Individuals with a deficiency in complement components C5 to C8 are at increased risk of meningococcemia. Meningitis is characterized by an abrupt onset of frontal headache, stiff neck (nuchal rigidity), confusion, and photophobia. The fatality rate is 10% to 15%; an additional 10% to 20% have serious sequelae, such as neurologic complications or seizures. Other complications include arthritis, pericarditis, and pneumonia. Meningococcal pneumonia is usually caused by serogroup Y and affects older adults with underlying pulmonary problems. On rare occasions, *N. meningitidis* can cause conjunctivitis and urethritis.

Laboratory diagnosis

Specimen collection and transport

Specimens for the recovery of *N. meningitidis* may come from a wide variety of sterile and nonsterile sites. These include cerebrospinal fluid (CSF), blood, nasopharyngeal swabs and aspirates, joint fluids, and, less commonly, sputum and material from urogenital sites. Collection and transport should be performed as specified by the laboratory for the various specimen types. Because *N. meningitidis* is inhibited by SPS, when commercial blood culture systems are used, information from the manufacturer should be consulted to determine whether *N. meningitidis* can be recovered.

Direct microscopic examination

On Gram-stained smears from specimens, such as CSF, meningococci appear as intracellular and extracellular gram-negative diplococci (Fig. 17.9). The highest yield of positive results of CSF Gram stain is obtained when specimens are concentrated. At least 1 mL of CSF should be centrifuged at $\times 1000g$ for 10 minutes and the sediment used for slide preparation and culture. The use of a cytocentrifuge is recommended and increases the sensitivity for detection by concentrating small numbers of organisms 100-fold. In a patient who has disseminated meningococcemia with petechiae from hemorrhage of surface blood vessels, impression Gram stain is often positive for gram-negative diplococci.

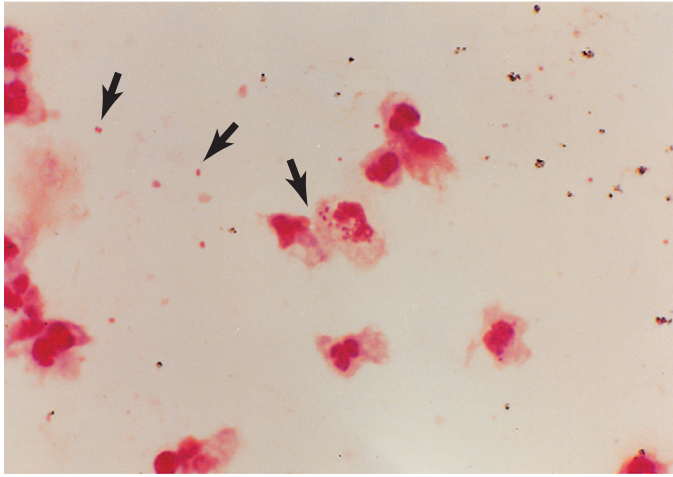


Fig. 17.9 Direct Gram-stained smear of cerebrospinal fluid illustrating intracellular and extracellular gram-negative diplococci (arrows) of *Neisseria meningitidis* (×1000).



Fig. 17.10 Growth of *Neisseria meningitidis* after 48 hours on chocolate (CHOC) agar (left) and sheep blood agar (SBA) (right). Of the *Neisseria* pathogens, only the meningococcus grows on SBA and CHOC agar.

Culture and incubation

Specimens for isolation of *N. meningitidis* should be cultured on SBA and CHOC agar. Specimens from mucosal surfaces that contain normal microbiota should also be inoculated to a selective medium, as described earlier for the culture of *N. gonorrhoeae*. Cultures should be incubated in the same atmospheric conditions described for *N. gonorrhoeae* and examined daily for 72 hours.

Identification

Presumptive identification is based on colony morphology, microscopic morphology, and the oxidase test. Colonies of meningococci usually grow within 18 to 24 hours and, in contrast with *N. gonorrhoeae*, grow well on SBA. The colonies are medium size, gray, and convex, and encapsulated strains are mucoid. The blood agar underneath the colonies tends to have a green tinge. *N. meningitidis* also grows on the selective agars, producing colorless to gray, convex, smooth colonies (Fig. 17.10).

N. meningitidis appears as gram-negative diplococci with adjacent sides flattened. Testing must be performed to confirm that the isolate is oxidase positive. Details of this test are described under *N. gonorrhoeae*. Definitive identification of *N. meningitidis* can be made with the use of carbohydrate methods, chromogenic enzyme tests, or multitest assays. In the carbohydrate utilization methods, *N. meningitidis* uses glucose and maltose. *N. lactamica*, generally a nonpathogenic species that may mimic *N. meningitidis*, can also grow on selective media. The lactose-positive characteristic of *N. lactamica* may be delayed or absent. A rapid *o*-nitrophenyl- β -D-galactopyranoside (ONPG) test, which detects lactose utilization, is usually positive in 30 minutes for *N. lactamica*. See Chapter 9 for a description of the ONPG test.

Although rare, *N. meningitidis*, as well as *N. gonorrhoeae*, may fail to acidify carbohydrate-containing media. Maltose-negative strains of *N. meningitidis* could be misidentified as *N. gonorrhoeae*, especially if recovered from the genital tract or other unusual sites. Maltose-negative strains may lack the maltose phosphorylase pathway; this occurs mainly in serogroup B but also rarely in groups C and Y. To avoid misidentification of maltose-negative strains, enzyme tests and multitest methods can be utilized. One useful test included in some identification systems is γ -glutamyl aminopeptidase, which is usually positive for *N. meningitidis* and negative for *N. gonorrhoeae*, *N. lactamica*, and *M. catarrhalis*.

If testing identifies the isolate as *N. meningitidis*, serologic testing should be performed to determine the serogroup. Isolates can be sent to a reference or public health laboratory for serogrouping. Additional methods using molecular techniques and MALDI-TOF mass spectrometry are also available.

Laboratory-acquired disease

In 2000, two cases of fatal laboratory-acquired meningococcal disease were reported to the CDC. Both clinical microbiologists had examined plates, performed Gram stain, subcultured, or performed slide agglutination serogrouping on patient isolates on the open bench. Isolates recovered from the laboratory scientists were identical to patient organisms. A retrospective survey identified 16 probable laboratory-acquired meningococcal infections worldwide from 1996 to 2001. Because exposure to *N. meningitidis* aerosols increases risk of infection, the CDC recommends the use of a biosafety level 2 cabinet for manipulation of suspected isolates of *N. meningitidis* from sterile sites.

Treatment

Empiric therapy for suspected meningococcal disease consists of an extended-spectrum cephalosporin (cefotaxime or ceftriaxone). Upon confirmation of the microbiologic diagnosis, definitive treatment with penicillin G, ampicillin, or an extended-spectrum cephalosporin is recommended. Routine susceptibility of *N. meningitidis* was not previously recommended; however, recent reports of β -lactamase-producing *N. meningitidis* serogroup Y cases in the United States have prompted a recommendation that susceptibility to penicillin be determined before administering.

Close contacts of patients with invasive meningococcal disease are at increased risk of contracting this infection. Chemoprophylaxis with rifampin, ceftriaxone, or ciprofloxacin is recommended for contacts. **Azithromycin is not recommended as a first-line chemoprophylactic agent, but it can be used in areas where ciprofloxacin resistance is a problem. Chemoprophylaxis is not recommended for asymptomatic carriers.**

Vaccine

Three quadrivalent polysaccharide–protein conjugated vaccines with antigens to serogroups A, C, Y, and W-135 are available in the United States. These vaccines are licensed for use in individuals 2 to 55 years of age. The Advisory Committee on Immunization Practices recommends routine vaccination of individuals at 11 to 12 years of age, with a booster dose administered at 16 years of age. If not previously vaccinated, individuals entering high school and college freshmen living in dormitories should receive this vaccine. Meningococcal vaccine is also recommended for military recruits, patients with asplenia who are older than 2 years of age, and laboratory scientists who work with *N. meningitidis*. Individuals who receive their first dose at 16 years of age or older do not need a booster dose unless they are at a high risk of invasive meningococcal disease.

In 2014 and 2015, two vaccines against *N. meningitidis* serogroup B were approved. The capsular antigen B is weakly antigenic, and because of its similarity to human glycoproteins, it can cause hypersensitivity reactions as a result of molecular mimicry. The vaccines are conjugated vaccines of proteins from other bacteria bound to *N. meningitidis* group B outer membrane proteins. Vaccination is recommended for young adults 16 to 23 years of age and will provide short-term protection against 91% of meningococcal B strains in the United States. This vaccine is administered as a two- or three-dose series and is recommended for individuals at increased risk of meningococcal group B disease.

Moraxella catarrhalis

Clinical infections

M. catarrhalis, a commensal of the upper respiratory tract isolated only from humans, is an opportunistic pathogen. It is recognized as a cause of upper respiratory tract infection in otherwise healthy children and in older adults. This organism has been reported as the third most common cause of acute otitis media and sinusitis in children, following *Streptococcus pneumoniae* and *Haemophilus influenzae* (Fig. 17.11A). The exact pathogenesis of otitis media is not clear, but colonization and persistence in the nasopharynx is accepted to be the initiating step. The mechanism of transition from colonization to infection is not understood, but it is accepted that a viral upper respiratory infection causes changes in the host that leads to the onset of acute otitis media. Recent studies indicate that biofilms and polymicrobial infections are a significant factor in recurrent and chronic infections that are difficult to eradicate. *M. catarrhalis* is more often found in polymicrobial otitis media infections than from a single-species infection.

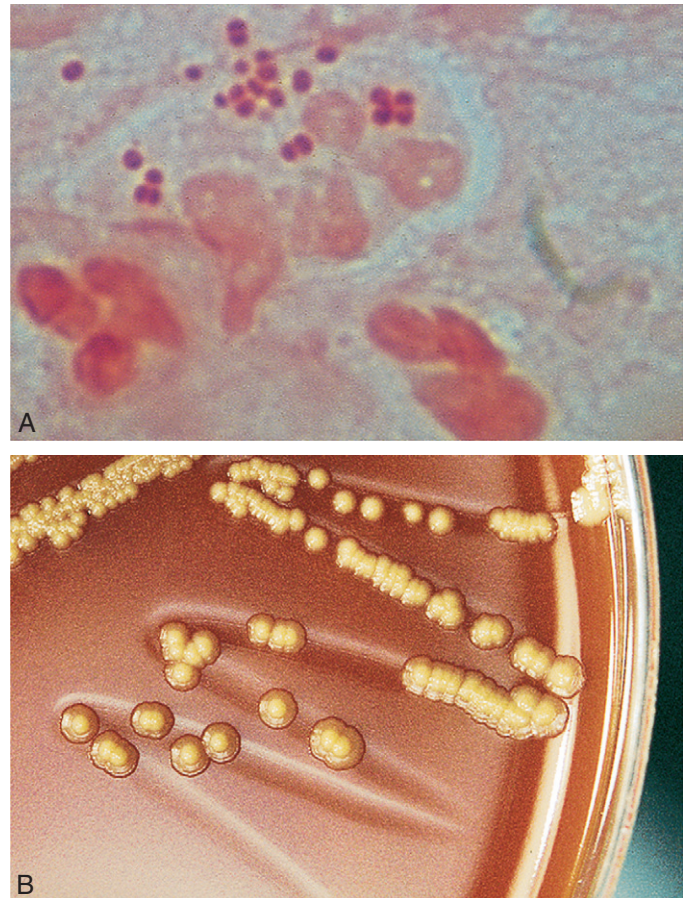


Fig. 17.11 **A**, Direct Gram-stained smear of an otitis media specimen illustrating intracellular, gram-negative diplococci ($\times 1000$). The organism was identified biochemically as *M. catarrhalis* from cultures. **B**, Growth of *Moraxella catarrhalis* after 48 hours, illustrating the “wagon-wheel” appearance on chocolate agar.

M. catarrhalis can also cause lower respiratory tract infections including bronchitis and pneumonia, especially in adults with chronic obstructive pulmonary disease. Other predisposing factors include advanced age, immunodeficiency, neutropenia, and chronic debilitating diseases. Additional rare infections include life-threatening systemic diseases, such as endocarditis, meningitis, and bacterial tracheitis. Severe infections are seen in immunocompromised hosts; hospital outbreaks of *M. catarrhalis* respiratory infections have occurred. The presence of intracellular, gram-negative diplococci in these specimens may alert the microbiologist to possible infection with *M. catarrhalis*. Most isolates produce β -lactamase, making them resistant to ampicillin and amoxicillin. Antimicrobial agents used include amoxicillin-clavulanic acid, extended-spectrum cephalosporins, azithromycin, and trimethoprim-sulfamethoxazole.

Laboratory diagnosis

Specimen collection and identification

Typical specimens for *M. catarrhalis* can be collected from middle ear effusion, nasopharynx, sinus aspirates, sputum, or bronchial aspirates. The organism grows on SBA and CHOC

agar, producing smooth, opaque, gray-to-white colonies. The term *hockey puck* has been used to describe the colony because it remains intact when pushed across the plate with a loop. Older colonies may give a “wagon-wheel” appearance (see Fig. 17.11B). In contrast with *Neisseria* spp. that have an optimal growth temperature of 35° to 37° C, most strains of *M. catarrhalis* can tolerate lower temperatures and grow well at 28°C. *M. catarrhalis* is usually inhibited on gonococcal selective agars by colistin, but some strains resistant to this antimicrobial may grow. Similar to *Neisseria* spp., *M. catarrhalis* is oxidase and catalase positive. The organism is asaccharolytic, and it may be differentiated from *Neisseria* spp. by positive DNase and butyrate esterase reactions. Tributyrin is used as the substrate to detect butyrate esterase activity. MALDI-TOF mass spectrometry provides rapid and accurate identification.

Commensal *Neisseria* species

Other *Neisseria* spp. exist as normal inhabitants of the upper respiratory tract. Referred to as *commensals*, *saprophytes*, or *nonpathogens*, these species are occasionally isolated from the genital tract. The commensal *Neisseria* spp. rarely cause disease, but they have sporadically been implicated in meningitis, endocarditis, prosthetic valve infections, bacteremia, pneumonia, empyema, bacteriuria, osteomyelitis, and ocular infections (Table 17.6). Many rapid tests for the identification

Table 17.6 Infections reported to be caused by <i>Neisseria</i> species other than <i>N. gonorrhoeae</i> and <i>N. meningitidis</i>	
Infection	<i>Neisseria</i> species
Meningitis	<i>N. lactamica</i>
	<i>N. sicca</i>
	<i>N. subflava</i>
	<i>N. mucosa</i>
	<i>N. flavescens</i>
Endocarditis	<i>N. sicca</i>
	<i>N. subflava</i>
	<i>N. mucosa</i>
	<i>N. elongata</i>
	<i>N. flavescens</i>
Prosthetic valve infection	<i>N. bacilliformis</i>
	<i>N. sicca</i>
Bacteremia	<i>N. lactamica</i>
	<i>N. flavescens</i>
Pneumonia	<i>N. cinerea</i>
	<i>N. sicca</i>
Empyema	<i>N. mucosa</i>
Bacteriuria	<i>N. subflava</i>
Osteomyelitis	<i>N. sicca</i>
Ocular infection	<i>N. cinerea</i>
Dog bite	<i>N. mucosa</i>
	<i>N. weaveri</i>

of *N. gonorrhoeae* test for a limited number of characteristics that may be shared by one or more nonpathogenic *Neisseria* spp. One must keep in mind that the incidence of infections caused by the commensal *Neisseria* spp. is extremely low and that the main reason for the clinical microbiologist to be familiar with these organisms is to accurately separate them from the pathogenic species.

Identification

Table 17.7 lists the colony morphology and primary isolation sites of the *Neisseria* spp. and related organisms. Table 17.8 lists the traditional and additional tests used to identify the *Neisseria* spp. and some similar genera. These organisms are divided into three groups as follows:

- Group 1: Traditional pathogens
 - Group 2: Commensal *Neisseria* spp. that can grow on selective media
 - Group 3: Commensal *Neisseria* spp. that do not usually grow on selective media
- Groups 2 and 3 are divided further on the basis of their activities toward carbohydrates.

In the clinical laboratory, when isolated from respiratory specimens, commensal *Neisseria* spp. are usually identified only with Gram stain and gross colony morphology and are called *Neisseria* spp. or *usual oral biota*. Further identification by biochemical tests is not done. When they are isolated from selective agar or sterile body sites, differentiation from the pathogenic *Neisseria* may be required. Common laboratory tests and observations do not always adequately differentiate all of the commensal species from one another or from the pathogens. In addition, insufficient test parameters and equivocal carbohydrate reactions have led to confusion between the pathogenic and commensal *Neisseria* spp. Additional tests used to help further differentiate these organisms include growth on nutrient agar at 35°C, growth on SBA or CHOC agar at 22°C, and the reduction of nitrate and nitrite.

Neisseria cinerea

N. cinerea has received considerable attention in recent years because of its misidentification as *N. gonorrhoeae* in some commercial identification systems. Although *N. cinerea* is glucose negative in CTA sugars, the glucose was interpreted as positive in some commercial kits, making it biochemically identical to *N. gonorrhoeae*. The colony morphology of *N. cinerea* is also similar to the T3 colonies of *N. gonorrhoeae* on CHOC agar. Some strains of *N. cinerea* will grow on gonococcal selective medium. *N. cinerea* will grow well on SBA (Fig. 17.12), whereas *N. gonorrhoeae* usually does not.

Colistin susceptibility is a helpful test in differentiating *N. cinerea* from *N. gonorrhoeae*. A suspension of the organism is swabbed onto an SBA or CHOC agar plate, a 10-μg colistin disk is applied to the inoculum, and the plate is incubated in CO₂ for 18 to 24 hours. *N. cinerea* is susceptible (≥10 mm zone of inhibition) to colistin, whereas *N. gonorrhoeae* is resistant. Useful tests for differentiation of *N. cinerea* from *M. catarrhalis* are reduction of nitrate and negative DNase reaction. Useful observation for differentiation from *Neisseria flavescens* is lack of yellow pigment production.

Table 17.7 Colony morphology and primary isolation sites of *Neisseria* and related organisms

Organism	Colony morphology ^a	Primary isolation sites
<i>N. gonorrhoeae</i>	Small (0.5–1 mm), grayish white, translucent, raised with entire edge Usually easily emulsified Smaller than <i>N. meningitidis</i> Up to five different colony morphologies from primary culture	Male: urethra Female: endocervix Laboratory should be notified to look for this organism from other sites so appropriate media can be used
<i>N. meningitidis</i>	1–2 mm, bluish gray or tan (serogroup 6 may be yellowish) Serogroups A and C may be mucoid, translucent, and convex with smooth glistening surface; may be greenish cast in agar around colonies Usually easily emulsified	Nasopharynx and oropharynx (carriers) Spinal fluid: meningitis Blood: meningococcemia Lower respiratory tract: meningococcal pneumonia
<i>N. polysaccharea</i>	Small, gray (sometimes yellowish), translucent, raised Resembles <i>N. gonorrhoeae</i> colony	Nasopharynx of infants and children
<i>N. lactamica</i>	Small, grayish white (often with a yellow ring), translucent, slightly butyrous Resembles <i>N. meningitidis</i> but smaller	Nasopharynx of infants and children Rarely found in adults
<i>N. cinerea</i>	Small (1–1.5 mm), grayish white, translucent, raised with entire edge and slightly granular Resembles <i>N. gonorrhoeae</i>	Nasopharynx
<i>N. mucosa</i>	Large (up to 4 mm), grayish to buff yellow, translucent and mucoid, smooth surface, entire edge Viscous (sticky) consistency	Nasopharynx
<i>N. sicca</i>	Large (up to 3 mm), grayish white, opaque, deeply wrinkled, dry, irregular (breadcrumb-like) colony Firmly adherent, difficult to impossible to emulsify	Nasopharynx, saliva, sputum
<i>N. subflava biovars</i>	0.5–2 mm, pale greenish yellow to yellow, smooth surface with entire edge Often adherent	Nasopharynx
<i>N. flavescens</i>	Colonies similar to <i>N. meningitidis</i> but with golden yellow pigment	Pharynx (not often isolated from clinical specimens)
<i>N. elongate</i>	Large (up to 3 mm), grayish white with yellowish tinge, low convex to almost flat Corroding of agar may occur Claylike colony, difficult to emulsify	Nasopharynx
<i>N. weaver</i>	Small, semi-opaque with smooth appearance	Wounds from dog bites
<i>N. bacilliformis</i>	Small, smooth, glistening, light gray to buff	Upper respiratory tract
<i>Kingella denitrificans</i>	Small (1–2 mm), gray, semi-transparent, convex, may pit the agar Colony morphology of organisms that do not pit the agar similar to <i>N. gonorrhoeae</i>	Upper respiratory tract
<i>Moraxella catarrhalis</i>	3–5 mm, grayish white, opaque 48-hour colony may have elevated center and thinner, wavelike periphery ("wagon wheel") Often granular, difficult to emulsify Colony can be swept across plate intact ("hockey puck")	Upper respiratory tract

^aOn chocolate agar at 24 to 48 hours.

Table 17.8 Differential tests for commensal *Neisseria* and related genera

Organism	Traditional tests								Additional tests				
	Growth on ML, MTM, or NYC	Catalase	Oxidase	Glucose	Maltose	Acid produced from			Growth on blood or chocolate agar at 22° C	Growth on nutrient agar at 35° C	Reduction of		
						Lactose (ONPG)	Sucrose	Fructose			Nitrate	Nitrite	DNase
Group 1: traditional pathogens													
<i>N. gonorrhoeae</i>	+	+	+	+	–	–	–	–	–	–	–	–	–
<i>N. meningitidis</i>	+	+	+	+	+	–	–	–	–	–	–	V	–
Group 2: commensal species—possible growth on selective agar media													
Saccharolytic													
<i>K. denitrificans</i>	V	–	+	(+)	–	–	–	–	+	+	+	V	–
<i>N. lactamica</i>	+	+	+	+	+	+	–	–	V	+	–	V	–
<i>N. polysaccharea</i>	V	+	+	+	+	–	V	–	–	+	–	V	–
Asaccharolytic													
<i>N. cinerea</i>	V	+	+	–	–	–	–	–	–	+	–	+	–
<i>M. catarrhalis</i>	V	+	+	–	–	–	–	–	V	+	+	+	+
Group 3: Commensal species—no growth on selective agar media													
Saccharolytic													
<i>N. mucosa</i>	–	+	+	+	+	–	+	+	+	+	+	+	
<i>N. sicca</i>	–	+	+	+	+	–	+	+	+	+	–	+	–
<i>N. subflava</i> bv. <i>subflava</i>	V	+	+	+	+	–	–	–	+	+	–	V	–
<i>N. subflava</i> bv. <i>flava</i>	V	+	+	+	+	–	–	+	+	+	–	V	–
<i>N. subflava</i> bv. <i>perflava</i>	V	+	+	+	+	–	+	+	+	+	–	V	–
Asaccharolytic													
<i>N. flavescens</i>	–	+	+	–	–	–	–	–	+	+	–	+	–
<i>N. elongata</i>	–	–	+	–	–	–	–	–	+	+	V	+	–
<i>N. weaveri</i>	–	+	+	–	–	–	–	–	+	+	–	+	–
<i>N. bacilliformis</i>	–	V	+	–	–	–	–	–	+		V	V	

+, Positive; –, negative; (+), positive (delayed); bv, biovar; DNase, deoxyribonuclease; ML, Martin-Lewis agar; MTM, modified Thayer-Martin agar; NYC, New York City medium; ONPG, o-nitrophenyl-β-D-galactopyranoside; V, variable.

+, Positive; –, negative; (+), positive (delayed); bv, biovar; DNase, deoxyribonuclease; ML, Martin-Lewis agar; MTM, modified Thayer-Martin agar; NYC, New York City medium; ONPG, o-nitrophenyl-β-D-galactopyranoside; V, variable.

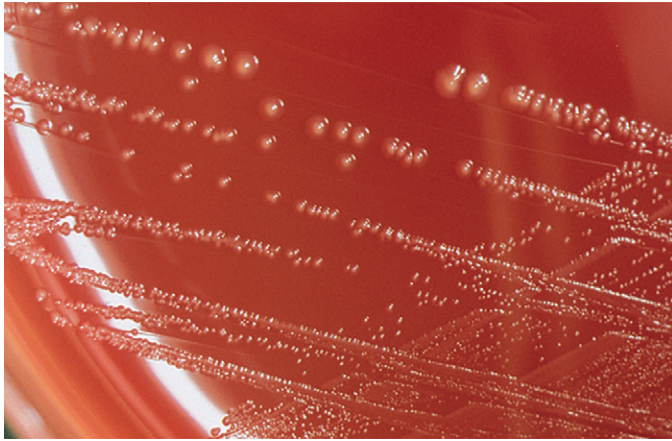


Fig. 17.12 Colony morphology of *Neisseria cinerea* on sheep blood agar (48-hour culture).



Fig. 17.13 Culture of *Neisseria lactamica* after 48 hours on sheep blood agar (left) and chocolate agar (right). This organism resembles *Neisseria meningitidis*.

Neisseria flavescens

N. flavescens (*flavescens* means “yellow”) is a yellow-pigmented *Neisseria* species that is asaccharolytic. It can be differentiated from *N. cinerea* by its ability to grow on SBA or CHOC agar at 22° C and its yellow colonies.

Neisseria lactamica

N. lactamica is commonly found in the nasopharynx of infants and children and, similar to *Neisseria polysaccharea*, is commonly encountered in meningococcal carrier surveys. The carriage rate of this species in children appears to peak at about 2 years of age and then steadily declines. It is rarely isolated from adults. It is the only *Neisseria* species that uses lactose—hence its species designation *lactamica*.

N. lactamica can be misidentified as *N. meningitidis* because of its similar colony morphology (*N. lactamica* is slightly smaller) (Fig. 17.13) and its ability to grow on selective media. Although *N. lactamica* uses glucose, maltose, and lactose, it can exhibit delayed lactose utilization and be confused with *N. meningitidis*. The definitive test for differentiation from *N. meningitidis* and all other *Neisseria* spp. is lactose utilization or positive ONPG reaction.

Neisseria mucosa

Colonies of *N. mucosa* are large, often adherent to the agar, and very mucoid, giving the species its name. *N. mucosa* is usually isolated as a nonpathogen from the nasopharynx of children or young adults. This organism has been documented to cause pneumonia in children. In addition, it has been associated with bacteremia and endocarditis in patients with neutropenia. It has the same carbohydrate pattern as *N. sicca* and *N. subflava* biovar *perflava*, but it differs from these species in its ability to reduce nitrite to nitrogen gas, its colony morphology, and its lack of pigment production.

Neisseria sicca

The colonies of *N. sicca* are usually dry, wrinkled, adherent, and breadcrumb-like (Fig. 17.14). The Latin word *sicca* means “dry.” *N. sicca* and *N. subflava* biovar *perflava* are usually the

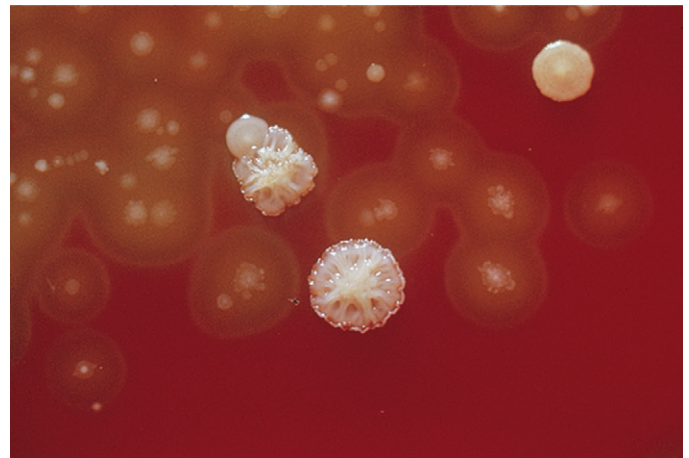


Fig. 17.14 Dry, wrinkled, breadcrumb-like colony morphology of *Neisseria sicca* on sheep blood agar (48-hour culture).

two most common *Neisseria* spp. found in the respiratory tract of adults. It has been reported to cause endocarditis.

Neisseria subflava

The species name for *N. subflava* means “less yellow” (Fig. 17.15). The organism is considered part of the upper respiratory microbiota. It consists of three biovars that differ from one another by their patterns of carbohydrate utilization. Although it is considered a nonpathogen, it has been reported to cause serious infections, such as bacteremia, meningitis, and septicemia. Its clinical description may resemble *N. meningitidis* infection, including septic shock, petechial hemorrhage, and purpura. It has reduced sensitivity to penicillin, cefixime, and ciprofloxacin.

Neisseria elongata

Neisseria elongata, *N. weaveri*, and *N. bacilliformis* are unique among the members of the genus *Neisseria* in that they are rod shaped. *N. elongata* contains three subspecies: *elongata*, *glycolytica*, and *nitroreducens*. The catalase test for all subspecies is

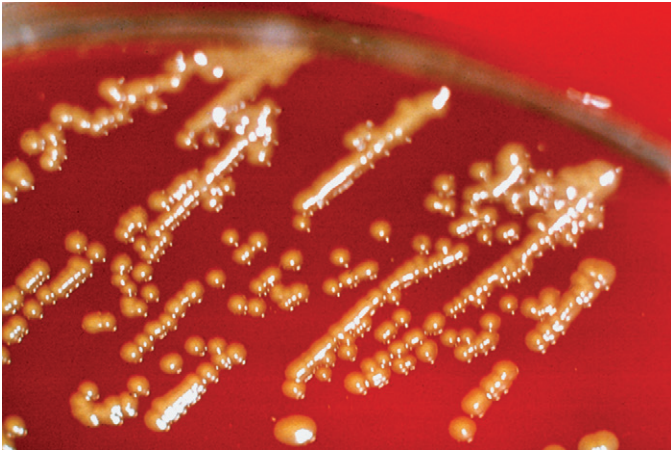


Fig. 17.15 Yellow pigmentation of *Neisseria subflava* on sheep blood agar (48-hour culture).

negative or weakly positive compared with other *Neisseria* spp. All three subspecies are commensals in the upper respiratory tract and are considered opportunistic pathogens. Several cases linking *N. elongata* to endocarditis have been reported.

Neisseria weaveri

N. weaveri is normal oral microbiota in dogs and can be found in humans in infections following dog bites. It is catalase positive and does not produce acid from any of the carbohydrates traditionally used to identify *Neisseria* spp. *N. weaveri* is a gram-negative rod that does not reduce nitrate but does reduce nitrite to gas. It is also weakly phenylalanine deaminase positive. Rare cases of septicemia in immunocompromised patients have been reported. This organism is usually sensitive to penicillin.

POINTS TO REMEMBER

- Most *Neisseria* spp. are gram-negative diplococci that occur in pairs, with adjacent sides flattened.
- Species of the genus *Neisseria* are oxidase-positive, aerobic organisms that grow best in increased CO₂ and humidity.
- *N. gonorrhoeae* and *N. meningitidis* are the primary human pathogens of the genus.
- All other *Neisseria* species are considered opportunistic pathogens and must be differentiated from *N. gonorrhoeae* and *N. meningitidis*.
- Pathogenic species are fastidious organisms requiring enriched and selective media for optimal recovery.
- Growth of the primary human pathogens on selective media, such as modified Thayer-Martin (MTM) or New York city agars, is not considered confirmatory. Gram stain of the colony, oxidase, and additional laboratory tests must be performed to confirm identification.
- *M. catarrhalis* is a commensal of the upper respiratory tract and is associated with opportunistic infections, including otitis media, sinusitis, and lower respiratory tract infections.
- *M. catarrhalis* is asaccharolytic, and it is differentiated from *Neisseria* spp. by being tributyrin hydrolysis positive or DNase positive.
- The incidence of infections caused by commensal *Neisseria* spp. is very low. They are not usually identified unless found in a specimen from a normally sterile site and are rarely associated with septicemia and endocarditis.

LEARNING ASSESSMENT QUESTIONS

1. Which characteristics do most *Neisseria* species have?
 - a. Oxidase-positive, gram-positive diplococci
 - b. Oxidase-positive, gram-negative diplococci
 - c. Oxidase-negative, gram-negative diplococci
 - d. Oxidase-negative, gram-positive diplococci
2. Which virulence factor of the pathogenic *Neisseria* spp. is responsible for the initial attachment of the organism to host tissues?
 - a. Pili
 - b. Endotoxin
 - c. Cell membrane proteins
 - d. Peptidoglycan
3. Asymptomatic gonococcal infections in women may result in which condition?
 - a. Pelvic inflammatory disease
 - b. Ectopic pregnancy
 - c. Fitz-Hugh–Curtis syndrome
 - d. All of the above
4. Which is the optimal specimen to collect for the diagnosis of gonorrhea by culture in male patients?
 - a. Pharyngeal swab
 - b. Rectal swab
 - c. Urethral swab
 - d. Urine
5. Which of the following is (are) the reason(s) direct Gram stain is performed for the diagnosis of gonorrhea?
 - a. Is appropriate on vaginal specimens
 - b. Can accurately detect urethritis in males
 - c. Can accurately diagnose gonococcal pharyngitis
 - d. All of the above
6. Which selective medium allows the isolation of *N. gonorrhoeae* and *N. meningitidis*?
 - a. Phenylethyl alcohol blood agar
 - b. Chocolate (CHOC) agar
 - c. Columbia nalidixic acid
 - d. Modified Thayer-Martin (MTM) agar
7. Which test can be used for definitive identification of both *N. gonorrhoeae* and *N. meningitidis*?
 - a. Gram stain
 - b. Catalase
 - c. Oxidase
 - d. Rapid carbohydrate utilization
8. What is the advantage of nucleic acid amplification tests for diagnosing gonorrhea?
 - a. Are approved for use in testing in boys in cases of sexual abuse
 - b. Detect viable organisms in the specimen
 - c. Are sensitive and do not require invasive specimens
 - d. Require strict transport conditions

9. What are the current recommendations for treatment of genital gonorrhea?
10. Which opportunistic pathogen is asaccharolytic, tributyrin hydrolysis positive, and DNase positive?
 - a. *Kingella denitrificans*
 - b. *Neisseria mucosa*
 - c. *Neisseria lactamica*
 - d. *Moraxella catarrhalis*
11. Entrance of *N. meningitidis* into the bloodstream may lead to which condition?
 - a. Meningococcemia
 - b. Meningitis
 - c. Waterhouse-Friderichsen syndrome
 - d. All of the above
12. Which test can accurately differentiate *N. lactamica* from *N. meningitidis*?
 - a. ONPG
 - b. Catalase
 - c. Acid from maltose
 - d. Acid from sucrose
13. Which organism is an opportunistic pathogen associated with otitis media and sinusitis in children?
 - a. *Moraxella catarrhalis*
 - b. *Neisseria lactamica*
 - c. *Neisseria meningitidis*
 - d. *Neisseria sicca*
14. Describe the colony morphology of *M. catarrhalis*, and explain how it is identified in the laboratory.
15. Erythromycin eye drops are placed into the eyes of newborns to prevent infections caused by which organism?
 - a. *Neisseria meningitidis*
 - b. *Neisseria gonorrhoeae*
 - c. *Neisseria cinerea*
 - d. *Moraxella catarrhalis*

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Haemophilus, HACEK group, *Legionella*, and other fastidious gram-negative bacilli

Tori Enomoto and A. Christian Whelen

CHAPTER OUTLINE

***Haemophilus*, 391**

General characteristics, 391

***Haemophilus influenzae*, 392**

Historic perspective, 392

Infections associated with other *Haemophilus* species, 394

Laboratory diagnosis, 394

Treatment, 397

HACEK group, 399

Aggregatibacter aphrophilus, 399

Aggregatibacter actinomycetemcomitans, 399

Cardiobacterium hominis, 401

Eikenella corrodens, 402

Kingella, 402

***Capnocytophaga*, 403**

***Pasteurella*, 404**

***Brucella*, 404**

***Francisella*, 406**

***Legionella*, 407**

General characteristics, 407

Virulence factors, 407

Infections caused by *Legionella*, 408

Epidemiology, 408

Laboratory diagnosis, 408

Specimen collection and handling, 409

Microscopic examination, 409

Isolation and identification, 409

Serologic testing, 411

Antimicrobial susceptibility, 411

***Bordetella*, 411**

General characteristics, 412

Clinically significant species, 412

Miscellaneous species, 412

Laboratory diagnosis, 412

Specimen collection and handling, 413

Nucleic acid detection, 413

Isolation and identification, 413

Serologic testing, 413

Antimicrobial susceptibility, 414

Bibliography, 416

OBJECTIVES

After reading and studying this chapter, you should be able to:

1. Distinguish each bacterial species discussed in this chapter by colony and microscopic morphology, habitat, nutritional requirements, and key identifying characteristics.
2. Compare the growth of the *Haemophilus* spp. colonies when performing tests for determination of X factor and V factor requirements.
3. Predict which antimicrobial agents to use in treating infections described in this chapter based on β -lactamase results.
4. Compare the pathogenesis, including modes of transmission, of each organism discussed.
5. Explain the clinical significance of the organisms discussed when isolated in the clinical laboratory.
6. Recommend the appropriate specimens for the recovery of these organisms.
7. Determine the appropriate culture media required for isolation of each organism.
8. Justify the use of nonculture laboratory methods used to detect these organisms.
9. Describe the genera and species of the organisms included in the acronym HACEK and the major diseases they cause.
10. Identify the most common animal reservoir for members of the genera *Pasteurella*, *Brucella*, and *Francisella*.
11. Associate the risk factors for acquiring legionellosis.
12. Compare Legionnaires' disease with Pontiac fever.
13. Discuss rapid assays useful in the detection of *Legionella* spp.
14. Describe the composition and efficacy of the vaccine for pertussis.

15. Develop an algorithm for the diagnosis of pertussis from specimen collection to identification of the causative agent.
16. Discuss the serologic response to *Bordetella pertussis* infection.

KEY TERMS

Adenylate cyclase toxin	HACEK
δ -Aminolevulinic acid (ALA)	Legionnaires' disease
Atypical pneumonia	Nontypable <i>H. influenzae</i> (NTHi)
Bipolar staining	Paroxysmal phase
Bordet-Gengou potato infusion agar	Pertussis
Buboes	Pertussis toxin (PT)
Buffered charcoal yeast extract (BCYE)	Pontiac fever
Buffered charcoal yeast extract with 0.1% α -ketoglutaric acid (BCYE α)	Porphyria
Capnophilic	Regan-Lowe transport medium
Catarrhal phase	Satellitism
Chancroid	Select biological agent
Convalescent phase	Suppurative
Filamentous hemagglutinin (FHA)	Tracheal cytotoxin
Genital ulcer disease (GUD)	V factor (nicotinamide adenine dinucleotide [NAD])
	Whooping cough
	X factor (hemin or hematin)
	Zoonoses

Case in point

A 59-year-old female stroke victim was confined temporarily to a respite nursing home because her immediate family was on a short vacation. On admission, she complained of a slight respiratory ailment. She awoke 3 days later with a stiff neck and a severe headache. Because of a high fever and other signs and symptoms of meningitis, she was transported to a nearby hospital emergency department, where a lumbar puncture was performed. Her cerebrospinal fluid (CSF) was cloudy, with an elevated protein concentration and a decreased glucose concentration, and more than 420 white blood cells (WBCs)/mL were noted; 92% were polymorphonuclear cells. Direct Gram-stained smear revealed many WBCs and many small, gram-negative bacilli; some appeared coccobacillary with clear halos around them. Cultures of CSF and blood produced heavy bacterial growth on chocolate (CHOC) agar but not on sheep blood agar (SBA) or MacConkey (MAC) agar.

Issues to consider

After reading the patient's case history, consider:

- The factors that would have predisposed the patient to meningitis
- Whether a vaccine for the etiologic agent in question is available
- The primary causes of meningitis in patients in this age group
- The characteristics of the microscopic morphology and growth patterns on laboratory media that help provide a presumptive identification

This chapter describes miscellaneous, fastidious, pleomorphic (many shapes), small, gram-negative bacilli. Most of these organisms require special nutrients for isolation and identification. *Haemophilus* spp. are facultative anaerobes and obligate parasites that are primarily adapted to

the respiratory tract of humans and other animals. One major exception is *Haemophilus ducreyi*, which causes the sexually transmitted disease (STD) **chancroid**. The genera *Haemophilus*, *Actinobacillus*, *Pasteurella*, and *Aggregatibacter* belong to the family Pasteurellaceae. Pasteurellaceae are characteristically gram-negative, pleomorphic, coccoid-shaped to rod-shaped cells that are nonmotile and facultatively anaerobic. They form nitrites from nitrates and are oxidase and catalase positive. Specific species of the genera *Haemophilus*, *Aggregatibacter*, *Cardiobacterium*, *Eikenella*, and *Kingella* have been grouped together under the acronym **HACEK** (first letter of each genus). They reside in the human oral cavity and nasopharynx, and some species have an enhanced capacity to cause endocarditis.

Other fastidious, gram-negative bacilli, including *Capnocytophaga*, *Brucella*, *Francisella*, *Legionella*, and *Bordetella* are discussed in this chapter. At the present time, 14 species of *Capnocytophaga* have been identified, and six of them inhabit the human oral cavity. The remaining species are primarily normal inhabitants of the oral cavities of dogs and cats. Many members of the genera *Pasteurella*, *Brucella*, and *Francisella* cause zoonoses (animal diseases that are transmitted to humans from the primary animal host).

The respiratory pathogens *Legionella* and *Bordetella* are aerobic organisms. *Legionella* are aquatic microorganisms that can thrive in a variety of environments, whereas *Bordetella* exists in the respiratory tract of humans and animals either as commensals or pathogens.

Haemophilus

General characteristics

The genus *Haemophilus* consists of gram-negative, pleomorphic coccobacilli or rods that can vary microscopically from small coccobacilli in direct smears of clinical material to long filaments occasionally seen in stained smears of colony growth. They are nonmotile and facultatively anaerobic, ferment carbohydrates, are generally oxidase and catalase positive, reduce nitrates to nitrites, and are obligate parasites on the mucous membranes of humans and animals. There are approximately 13 species of *Haemophilus*. The eight species associated with humans are *H. influenzae*, *H. parainfluenzae*, *H. haemolyticus*, *H. parahaemolyticus*, *H. paraphrohaemolyticus*, *H. pittmaniae*, *H. aegyptius*, and *H. ducreyi*. *H. segnis* was renamed *Aggregatibacter segnis*. Two other former members of the genus *Haemophilus*—*H. aphrophilus* and *H. paraphrophilus*—were also moved into the genus *Aggregatibacter* and combined into the single species *A. aphrophilus*. The *Aggregatibacter* spp. are also discussed in this chapter.

Most members of the genus *Haemophilus* are nonpathogenic or produce opportunistic infections. The emphasis of this section is on the major pathogenic species: *H. influenzae*, *H. aegyptius*, and *H. ducreyi*. *Haemophilus* is derived from the Greek word meaning “blood-lover.” As the name implies, *Haemophilus* organisms require preformed growth factors present in blood: **X factor** (hemin or hematin; X for “unknown”), **V factor** (nicotinamide adenine dinucleotide [NAD]; V for “vitamin”), or both. Traditionally, a small, gram-negative bacillus or coccobacillus is assigned to this

genus based on its requirements for one or both of the growth factors. *Haemophilus* spp. with the prefix *para* require only V factor for growth.

The production of hemolysis on 5% horse or rabbit blood agar is an important differential characteristic. Although certain species are also hemolytic on SBA, the organisms do not grow in pure culture on this medium. Both X and V factors are found within red blood cells (RBCs); however, only X factor is directly available. *Haemophilus* spp. that are V factor dependent do not grow on SBA because RBCs are still intact, and the sheep RBCs contain enzymes (NADases) that hydrolyze V factor. Clinical laboratories use chocolate (CHOC) agar for the recovery of *Haemophilus* spp. from clinical specimens. The lysing of the RBCs by heat in the preparation of CHOC agar releases both X factor and V factor and inactivates NADases. Supplemental nutrients are added.

A phenomenon that helps in the recognition of *Haemophilus* spp. that require V factor is **satellitism**. Satellitism occurs when an organism, such as *Staphylococcus aureus*, *Streptococcus pneumoniae*, or *Neisseria* spp., produces V factor as a by-product of metabolism. The *Haemophilus* isolate obtains X factor from the SBA and V factor from one of these organisms. On SBA plates, tiny colonies of *Haemophilus* may be seen growing around the V factor-producing organism. Fig. 18.1 illustrates *H. influenzae* satellitism around colonies of *S. aureus*. Except for *H. ducreyi*, all clinically significant *Haemophilus* spp. require V factor for growth and display this unusual growth pattern.

The indigenous microbiota of the healthy upper respiratory tract consists of many different genera and species of bacteria (see Chapters 2 and 32). Approximately 10% of this normal bacterial biota in adults consists of *Haemophilus* spp., with most of the organisms being *H. parainfluenzae* and, to a lesser extent, non-encapsulated *H. influenzae* that combined are carried by greater than 90% of healthy individuals. Colonization by *Haemophilus* spp. begins in infancy.

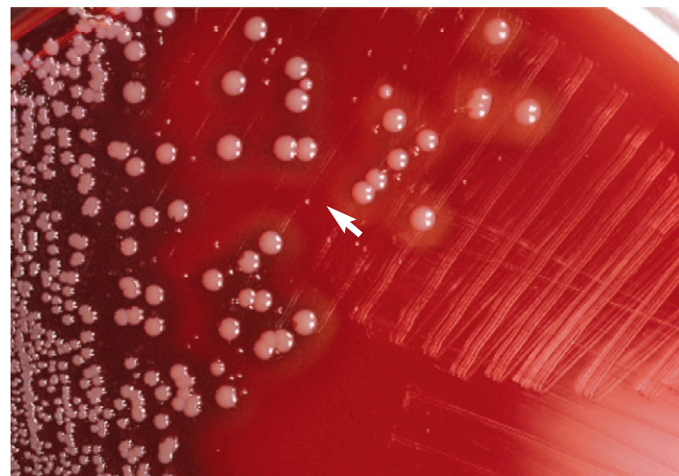


Fig. 18.1 *Haemophilus influenzae* satellitism around and between large, white, hemolytic staphylococci. The small, gray glistening colony is *H. influenzae* (arrow).

Haemophilus influenzae

Historic perspective

Influenza, commonly referred to as the *flu*, is a viral disease characterized by acute inflammation of the upper airways. Symptoms often progress to intense inflammation of the mucous membranes lining the nose (coryza), headache, bronchitis, and severe generalized muscle pain (myalgias). *H. influenzae* was erroneously named during the influenza pandemic that ravaged the world from 1889 to 1890. The basis for this assumption was the frequent isolation of this bacillus from the nasopharynx of patients with influenza and from postmortem lung cultures (viral isolation methods were unavailable). After viral culture techniques were developed, it became apparent that influenza was caused by a virus and that the actual role of *H. influenzae* was that of a secondary (opportunistic) invader.

Virulence factors

H. influenzae, the major pathogen within the genus, has a wide range of pathogenic potential. The following virulence factors play a role in the initiation of infection and the invasiveness of this organism:

- Capsule
- Immunoglobulin A (IgA) proteases
- Adherence by fimbriae and other structures
- Outer membrane proteins and lipopolysaccharide (LPS)

Capsule

Of all of the virulence factors, the capsule, if present, plays the most significant role. The serologic grouping of *H. influenzae* into six antigenically distinct types—a, b, c, d, e, and f—is based on differences in the capsular polysaccharide. Before widespread use of a vaccine, most invasive infections were caused by the encapsulated strain *H. influenzae* serotype b (Hib) and occurred primarily in young children. Serotype b strains are rarely seen now in children in countries using the vaccine; however, occasional serious invasive infections are seen in adults, especially in those older than 65 years of age. In unvaccinated children, type b is a leading cause of meningitis. In contrast with the other serotypes, the serotype b capsule is a unique polymer composed of ribose, ribitol, and phosphate (polyribitol phosphate). Evidence suggests that the antiphagocytic property and anticomplementary activity of the type b capsule are important factors in virulence and the pathogenesis of invasive disease. Not all strains of *H. influenzae* are encapsulated. These strains are commonly referred to as **nontypable *H. influenzae* (NTHi)**.

Immunoglobulin A proteases

Secretory IgA is present on human mucosal surfaces of the respiratory tract, areas for which *H. influenzae* has a predilection. IgA can bind to bacteria and block their attachment. *H. influenzae* is the only member of the genus that produces IgA protease. Because this enzyme can cleave secretory IgA, its production can contribute to the virulence of the organism.

Adherence mechanisms

The role of adherence mechanisms as virulence factors for *H. influenzae* is not well defined. Studies indicate that most NTHi strains are adherent to human epithelial cells, whereas most serotype b strains are not. The lack of this adherent capability in type b organisms may explain the tendency for type b strains to cause systemic infections. The presence of this adherent capability by NTHi strains may explain the tendency for these strains to cause more localized infections, such as acute conjunctivitis.

Outer membrane components

Although the role of outer membrane proteins and LPS is not well defined, antibodies directed against these antigens can play a significant role in human immunity. Each one of these components may be responsible for a specific activity, such as invasiveness, attachment, and antiphagocytic function. LPS has been shown to have a paralyzing effect on the sweeping motion of ciliated respiratory epithelium.

Clinical manifestations of Haemophilus influenzae infections

Two patterns of disease are attributed to *H. influenzae*. The first is invasive disease caused by encapsulated strains, in which bacteremia plays a significant role. Examples of invasive disease include septicemia, meningitis, arthritis, epiglottitis, tracheitis, and pneumonia. The second and the more common pattern of disease, as a result of Hib vaccination, is a more localized infection caused by the contiguous spread of NTHi strains, and it occurs within or in close proximity to the respiratory tract. Examples of localized infection include conjunctivitis, sinusitis, and otitis media with effusion (middle ear infections). *H. influenzae*, along with *Streptococcus pneumoniae*, is a common cause of middle ear infections in children. It has been demonstrated that the adenoids can serve as reservoirs in chronic middle ear infections and that the bacteria develop biofilms.

NTHi strains are also occasionally associated with invasive diseases, such as bronchitis and pneumonia, in older adult patients. High-risk factors include smoking, chronic obstructive pulmonary disease, and concurrent viral or bacterial infection. In contrast with encapsulated strains of *H. influenzae*, the NTHi strains can enter the central nervous system (CNS) by direct extension through infected sinuses, otitis media, and head trauma. Exceptions to the diseases caused by the encapsulated and NTHi strains do occur. NTHi strains can cause meningitis in adults, especially in immunocompromised or debilitated patients, and they can cause neonatal sepsis.

Before the advent of the Hib vaccine, the other serotypes rarely caused invasive disease in humans. Reports of infections have included pneumonia and bacteremia caused by serotypes a, d, and f in immunocompromised adults and neonatal sepsis caused by serotype c. Because of the widespread use of the Hib vaccine, hospital laboratories have reported a significant decrease in invasive disease among children, and diseases that do occur are generally caused by serotypes other than b. NTHi strains are currently responsible for most *H. influenzae* diseases in the United States. Data collected from the United States indicate that 70% of invasive *H. influenzae* infections were caused by NTHi. Serotype f (18%) accounted for the majority of invasive infections. Incidence is greatest among adults aged more than 65 years and children less than 1 year of age. The U.S. Centers

for Disease Control and Prevention (CDC) estimates that 6100 invasive case of *H. influenzae*, and 1015 deaths occur annually. In developing countries, where there is a lack of vaccination, serotype b is still a significant cause of bacterial pneumonia deaths and meningitis in children. *H. influenzae* remains a problem in older and debilitated people who have not been vaccinated. Table 18.1 summarizes infections caused by *H. influenzae*.

Case check 18.1

The older adult patient in the Case in Point had signs and symptoms of meningitis. The causative agent was a gram-negative bacillus that grew on CHOC agar but not on SBA or MAC agar. These characteristics strongly suggest *H. influenzae*. Determination of X factor and V factor requirement could confirm the diagnosis. Because of the routine use of a vaccine for *H. influenzae* type b, most invasive *H. influenzae* cases are caused by nontypable strains.

Meningitis

Before widespread use of the Hib vaccine, virtually all cases of meningitis caused by *H. influenzae* in children between 3 months and 6 years of age were caused by serotype b. Bloodstream invasion and bacteremic spread follow colonization, invasion, and replication of this organism in the respiratory mucous membranes. Headache, stiff neck, and other meningeal signs are usually preceded by mild respiratory disease.

Epiglottitis

The manifestations of epiglottitis include rapid onset, acute inflammation, and intense edema of the epiglottis that may cause complete airway obstruction, requiring an emergency tracheostomy. To avoid causing further possible damage, the area is not swabbed for culture but is treated empirically based on signs and symptoms. The peak incidence occurs in children between 2 and 4 years of age. Maintenance of a secure airway is the most important aspect of treatment.

Bacterial tracheitis

Similar to epiglottitis, bacterial tracheitis is a serious life-threatening disease in young children. It can arise after an acute, viral respiratory infection. Initially there is mild to moderate illness for approximately 2 to 7 days, but it progresses rapidly. Use of broad-spectrum antimicrobial agents during the early stages of the disease is imperative because thick secretions can occlude the trachea, so the disease must be differentiated from epiglottitis.

Table 18.1 Infections caused by *Haemophilus influenzae*

Encapsulated strains	Non-encapsulated strains
Septicemia	Otitis media with effusion
Septic arthritis	Conjunctivitis
Meningitis	Sinusitis
Osteomyelitis	Bacteremia
Cellulitis	Pneumonia ^a
Pericarditis	
Pneumonia	
Epiglottitis	

^aNontypable (non-encapsulated) strains cause lower respiratory tract infections primarily in older patients and individuals with underlying respiratory tract problems, including cystic fibrosis.

Infections associated with other *Haemophilus* species

Haemophilus aegyptius

H. aegyptius (Koch-Weeks bacillus) is genetically related to *H. influenzae*. Because of their similar identifying characteristics, it is difficult to differentiate *H. influenzae* from *H. aegyptius* and *H. influenzae* biogroup *aegyptius* in the clinical laboratory. *H. aegyptius* was observed by Koch in 1883 in Egyptians in conjunctivitis exudates—hence the species name. *H. aegyptius* is associated with an acute, contagious conjunctivitis, commonly referred to as *pink eye*.

Haemophilus influenzae biogroup *aegyptius*

H. influenzae biogroup *aegyptius* and *H. aegyptius* can cause conjunctivitis, primarily in pediatric populations. Despite being non-encapsulated, a clone of *H. influenzae* biogroup *aegyptius* first caused a severe systemic disease known as *Brazilian purpuric fever* (BPF) in Brazil in 1984. Currently, small outbreaks occasionally occur, primarily in South America. BPF is characterized by recurrent or concurrent conjunctivitis, followed by a sudden onset of high fever, petechial/purpurial rash, septicemia, shock, and vascular collapse. The mortality rate for BPF may reach 70% within 48 hours after onset.

Haemophilus ducreyi

H. ducreyi, a strictly human pathogen, is the causative agent of chancroid, a highly communicable sexually transmitted **genital ulcer disease (GUD)**. It infects the mucosal epithelium, genital and nongenital skin, and regional lymph nodes. Chancroid is commonly referred to as *soft chancre*, in contrast with the *hard chancre* of syphilis. Because chancroid facilitates the transmission of other STDs, all patients who have GUD should also be tested for human immunodeficiency virus (HIV), syphilis, and herpesvirus.

In contrast with other *Haemophilus* spp., this organism is not part of the normal microbiota. After an incubation period of approximately 4 to 14 days, a nonindurated, painful lesion with an irregular edge develops, generally on the genitalia or perianal areas. The most common sites of infection are on the penis or the labia or within the vagina. **Suppurative** (pus-forming), enlarged, draining, inguinal lymph nodes (**buboes**) are common in most infected patients (Fig. 18.2). Men have symptoms related to the inguinal tenderness and genital lesions, whereas most women are asymptomatic. Although less than 15 cases of chancroid are reported in the United States annually, as with *Neisseria gonorrhoeae* and *Chlamydia trachomatis*, cases are probably underreported. *H. ducreyi* is an important cause of GUD in Latin America, Africa, and Asia.

Miscellaneous species

H. parainfluenzae and the other species found in the oral cavity have a very low incidence of pathogenicity. *H. parainfluenzae* probably causes a few cases of otitis media and acute sinusitis and, although rare, has been implicated in cases of endocarditis. *H. parainfluenzae*-related endocarditis is a disease of insidious onset (i.e., a progressing disease without marked



Fig. 18.2 Lesions of chancroid on the penis, showing a draining bubo (arrow) in the adjacent groin area. Chancroid is caused by *Haemophilus ducreyi*.

symptoms before becoming apparent); first symptoms appear approximately 1 month after routine dental procedures. The mitral valve is the primary site of infection. In the absence of other pathogens, *H. parahaemolyticus* may be a cause of some cases of pharyngitis.

Laboratory diagnosis

Specimen processing and isolation

Haemophilus spp. have been associated with many diseases in humans. Almost any specimen submitted for routine bacteriologic examination may harbor these organisms. Common sources include blood, CSF, middle ear exudate, joint fluids, upper and lower respiratory tract specimens, swabs from conjunctivae, vaginal swabs, and abscess drainage. For culture of the lower respiratory tract, bronchial washing is recommended. Except in the case of patients with cystic fibrosis, nasal and nasopharyngeal swab specimens have no clinical value in evaluation for respiratory tract infections caused by *H. influenzae*.

Haemophilus spp. die rapidly in clinical specimens, and prompt transportation and processing are vital for their isolation and recovery. This is especially true of genital specimens submitted for *H. ducreyi*, which is extremely fastidious. Genital sites should first be cleansed with sterile gauze moistened with sterile saline before specimens are collected for the isolation of this organism. Next, a swab premoistened with sterile phosphate-buffered saline should be used to collect material from the base of the ulcer. As an alternative, pus can be aspirated from buboes if they are present. Direct plating on selective media at the bedside is preferred instead of using transport media. Because *Haemophilus* spp. are susceptible to drying, specimen processing in the laboratory should occur soon after collection for maximum recovery.

Because of the lack of one or more growth factors, most conventional media do not support the growth of *Haemophilus* spp. When attempting to isolate *H. influenzae*, CHOC agar is a commonly used medium incubated between 33° and 37° C in an atmosphere of 5% to 10% carbon dioxide (CO₂). It has been shown that CHOC agar supplemented with bacitracin (300mg/L) is an excellent medium for the isolation of *Haemophilus* spp. from respiratory specimens. Bacitracin is

added to reduce overgrowth of normal respiratory microbiota, a significant challenge to the isolation of *Haemophilus* spp. Growth on CHOC agar is usually seen after 18 to 24 hours of incubation.

Specimens submitted for *H. ducreyi* and *H. aegyptius* must be plated to special media because of their fastidious nature. For *H. aegyptius*, enriched CHOC agar supplemented with 1% IsoVitaleX (BD Diagnostic Systems, Sparks, MD) or Vitox (Oxoid, Basingstoke, UK) is required. Enriched CHOC medium is commonly used in clinical microbiology laboratories. *H. ducreyi* also grows on enriched CHOC medium, or a Nairobi biplate can be used. Nairobi biplate consists of GC agar base with 2% bovine hemoglobin and 5% fetal calf serum on one half and Mueller Hinton agar with 5% chocolate horse blood on the other. Both sides contain 3 mg/L of vancomycin. The use of GC agar (Gibco Laboratories, Grand Island, NY) containing 1% hemoglobin, 5% fetal calf serum, 1% IsoVitaleX, and 3 mg/L of vancomycin is also reported to be reliable. The use of vancomycin (most *H. ducreyi* are resistant) in the media helps reduce the growth of commensal biota from genital specimens and improves the detection of *H. ducreyi*. The plates for the recovery of this organism should be incubated in a 5% to 10% CO₂ atmosphere containing high humidity. In contrast with the optimal growth temperature (35° to 37° C) of the other *Haemophilus* spp., *H. ducreyi* grows best at 33° C. Cultures for *H. aegyptius* should be held for at least 4 days, and cultures for *H. ducreyi* should be held for at least 7 days before reporting a negative result.

Colony morphology

Most clinical specimens are plated onto a variety of culture media and examined after overnight incubation. Usually, SBA, CHOC agar, and MAC agar plates are inoculated simultaneously from clinical specimens from areas of the human body where *Haemophilus* organisms may be isolated. Colonies of *H. influenzae* on CHOC agar appear translucent, tannish, moist, smooth, and convex, with a distinct “mousy” or bleach-like odor. Fig. 18.3 shows the typical colony morphology of *H. influenzae*. The growth of *H. influenzae* biogroup *aegyptius* resembles *H. influenzae*. Encapsulated strains of *H. influenzae* grow larger and more mucoid than the NTHi strains. *Haemophilus* spp. do not grow on MAC agar. *Haemophilus* spp. sometimes grow on SBA plates around colonies of other bacterial species—a phenomenon known as *satellitism*, as discussed previously.

On CHOC agar, *H. parainfluenzae* colonies appear tannish and drier with a medium to large size compared with *H. influenzae*. On CHOC agar, *H. parahaemolyticus* resembles *H. parainfluenzae*; however, when grown on horse or rabbit blood agar, it is β -hemolytic, whereas *H. influenzae* is nonhemolytic. *H. ducreyi* appears as small, flat, smooth, nonmucoid, transparent to opaque colonies or appears tan or yellow on CHOC agar. Individual colonies can be pushed intact using a loop across the agar plate surface. They are difficult to pick up and produce a “clumpy” nonhomogeneous appearance when suspended in saline. Several publications have described in-house polymerase chain reaction (PCR) assays. The BioFire FilmArray (bioMérieux, Durham, NC) has a multiplex nucleic acid amplification meningitis/encephalitis panel for CSF that includes *H. influenzae*.

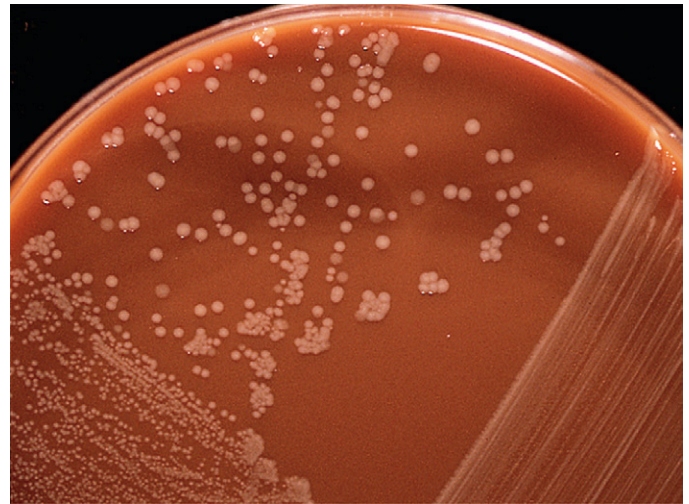


Fig. 18.3 Example of *Haemophilus influenzae* growing on chocolate agar. Note the tan mucoid colonies characteristic of encapsulated strains.

Microscopic morphology

With most *Haemophilus* spp., the microscopic morphology varies from small, gram-negative coccobacilli to long filaments (described as pleomorphic). The coccobacillary morphology is the more predominant form found in clinical specimens. Capsules of *H. influenzae* may be observed in Gram-stained direct smears as clear, nonstaining areas (“halos”) surrounding the organisms in purulent secretions. Because the organism is small and pleomorphic and often stains a faint pink, it can resemble the amorphous serous material (serumlike or proteinaceous background material) in Gram stains of clinical specimens.

Because of the low specificity and sensitivity of Gram stain, an acridine orange or methylene blue stain of the specimen may help detect *Haemophilus*. Fig. 18.4 illustrates the microscopic morphology of *H. influenzae* in a direct Gram-stained smear of CSF from a patient with meningitis. Fig. 18.5 is an example of a Gram-stained smear from an isolated colony of *H. influenzae*. Gram-stained smears for microscopic morphology of genital lesions or colonies for *H. ducreyi* may show pale-staining gram-negative coccobacilli arranged singly or in groups (clusters), commonly referred to as *school of fish* or *railroad tracks*, and loosely coiled clusters of organisms lined up in parallel or appearing as “fingerprints.”

Laboratory identification

The first clue that an isolate might belong to the genus *Haemophilus* is the growth of gram-negative pleomorphic coccobacilli in pure culture on CHOC agar, with no growth on SBA and MAC agar. Several tests can be used in the clinical laboratory for the identification of *Haemophilus* isolates, including testing for growth factors (X and V), oxidase, catalase, traditional biochemicals, and hemolysis on media containing rabbit or horse RBCs. In place of traditional biochemicals, several manual and automated commercial systems can be used to identify and biotype *Haemophilus* spp. within 4 hours. Recently, matrix-assisted laser desorption/ionization–time-of-flight

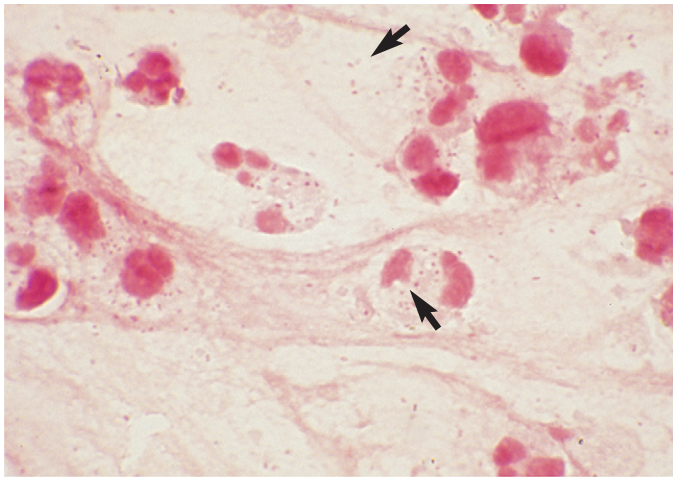


Fig. 18.4 Direct smear of *Haemophilus influenzae* in cerebrospinal fluid in a case of meningitis. Note the intracellular and extracellular gram-negative coccobacilli (arrows) ($\times 1000$).

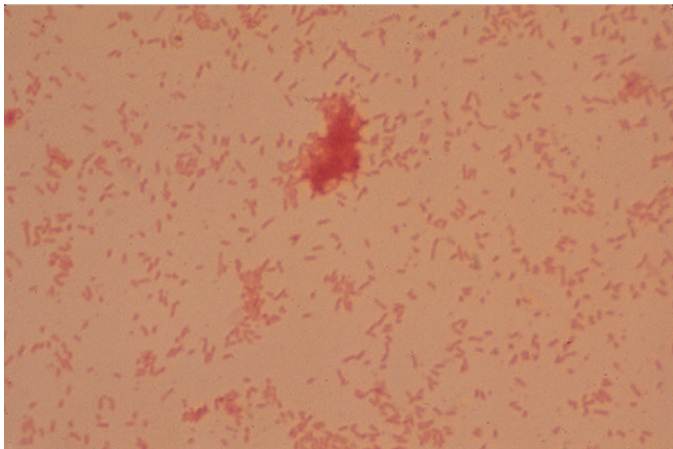


Fig. 18.5 Gram stain of a *Haemophilus influenzae* colony. Bacilli are slightly more elongated than in direct smears ($\times 1000$).

(MALDI-TOF) mass spectrometry has demonstrated excellent identification of the HACEK organisms.

X factor and V factor requirements

Testing for X factor and V factor requirements using impregnated strips or disks is the traditional approach for identification of *Haemophilus* spp. Care must be taken not to transfer any X factor-containing medium to the agar plates used for X factor requirement testing; carryover can produce erroneous or inconclusive results, causing *H. influenzae* to be misidentified as *H. parainfluenzae* (see [Appendix D](#) on Evolve). [Figs. 18.6 to 18.8](#) illustrate the reactions obtained when testing for X factor and V factor requirements using impregnated strips. When *Haemophilus* spp. are grown anaerobically, they do not require heme but still require NAD. If an *H. influenzae* isolate was incubated anaerobically in this test, it could be misidentified as *H. parainfluenzae*. Of all species that require V factor, *A. segnis* is the only organism that is oxidase negative.

The *Haemophilus* ID Quad Plate (Thermo Fisher Scientific, Waltham, MA) contains four zones: media with X factor only, with V factor only, with X and V factors, and with X and V factors with horse RBCs. The *Haemophilus* isolate may

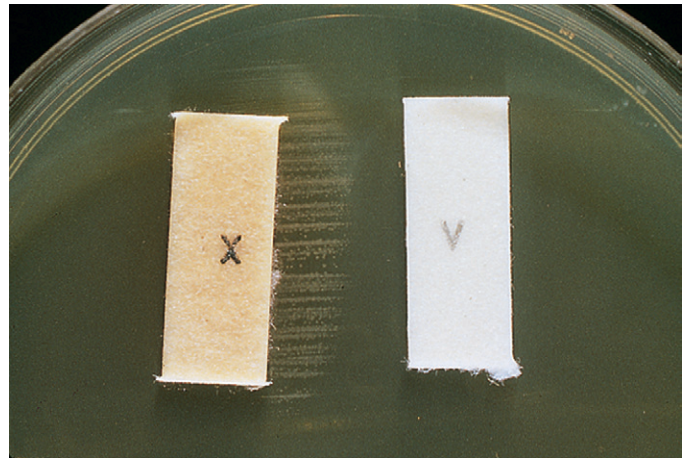


Fig. 18.6 This organism is identified as *Haemophilus influenzae* because it requires both X and V factors.

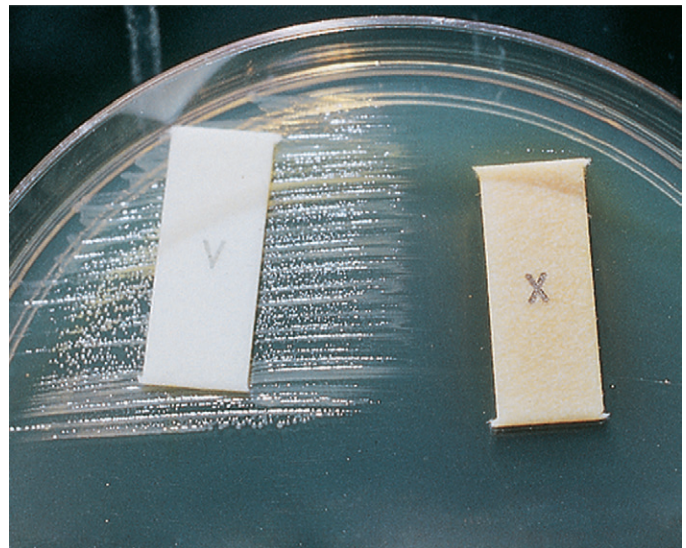


Fig. 18.7 This organism requires V factor only and is identified as *Haemophilus parainfluenzae*.

be identified based on the factors required for growth and the presence of hemolysis. *H. haemolyticus* is generally β -hemolytic on horse blood, whereas *H. influenzae* is negative. Misidentifying *H. haemolyticus* as *H. influenzae* may result in overtreatment because hemolysis is the major differentiating characteristic between the two species.

Porphyryn test

The **porphyrin** test is an alternative method for differentiating the heme-producing species of *Haemophilus*. The porphyrin test can be performed in agar, in broth, or on a disk. The principle of the test is based on the ability of the organism to convert the substrate δ -aminolevulinic acid (ALA) into porphyrins or porphobilinogen, which are intermediates in the synthesis of X factor. After incubation at 35° C for 4 hours, porphobilinogen is detected by the addition of *p*-dimethylaminobenzaldehyde (Kovac reagent). After addition of Kovac reagent, a red color forms in the lower aqueous phase if porphobilinogen is present. Porphyrins can be detected using an ultraviolet light with a wavelength of about 360 nm (Wood

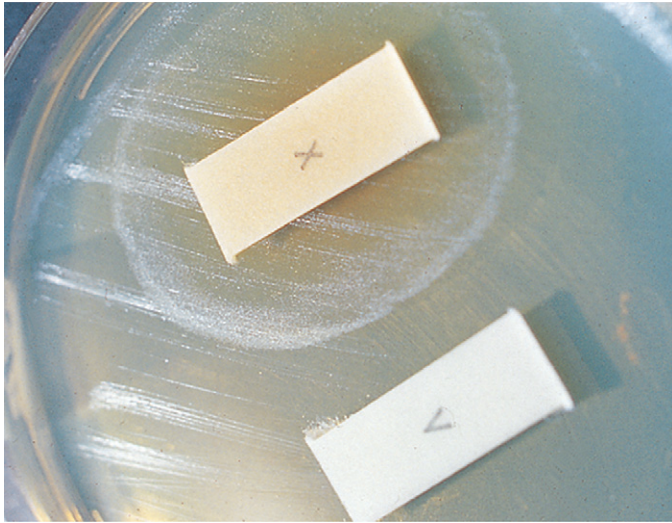


Fig. 18.8 This organism is positive for X factor only. The probable species is *Aggregatibacter aphrophilus* because this species can appear to be hemin dependent on initial isolation.

lamp). Porphyrins fluoresce to a reddish orange color under ultraviolet light.

The main advantage of the porphyrin test is that X factor is not required, and the problem of carryover is eliminated. The disadvantage is that primary identification is based on a negative test result. In the case of *H. influenzae*, the ultraviolet light test is negative (no fluorescence), and no color change occurs after addition of Kovac reagent. Species that are porphyrin negative cannot synthesize heme and are X factor positive (require hemin) when the impregnated strip is used. *Haemophilus* spp. that can synthesize heme are porphyrin positive (X strip negative, do not require hemin). **Fig. 18.9** illustrates the positive result of the porphyrin test performed on agar. The Clinical and Laboratory Standards Institute (CLSI) developed abbreviated test guidelines for the identification of commonly isolated bacteria. Gram-negative bacilli or coccobacilli isolated from respiratory tract specimens or CSF that exhibit colonies larger than 1 mm on CHOC agar and no growth or satellitism on SBA and a negative porphyrin test can be identified as *H. influenzae*.

Biochemical tests

Biochemical tests, such as carbohydrate fermentation, can help further differentiate *Haemophilus* spp. In addition, indole, urease, and ornithine decarboxylase tests are used to biotype some *Haemophilus* spp. Numerous commercial multitest systems are available, including the RapID NH system (Thermo Fisher Scientific), Crystal Neisseria/*Haemophilus* system (BD Diagnostic Systems), and NH ID Card (bioMérieux). **Table 18.2** lists the differential tests for *Haemophilus* and *Aggregatibacter* spp. **Table 18.3** lists the tests that differentiate *H. influenzae* and *H. parainfluenzae* biogroups. Differentiating the biogroups is generally necessary only in epidemiology studies.

Treatment

Invasive *H. influenzae* infection often requires hospitalization. The recommended treatment of life-threatening illness caused by *H. influenzae* is cefotaxime or ceftriaxone. Alternative drugs

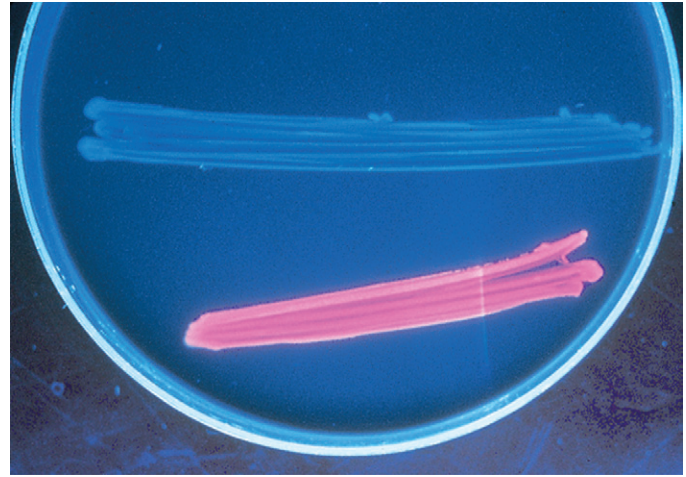


Fig. 18.9 Under ultraviolet light, the organism on the bottom is exhibiting a positive porphyrin reaction. The organism on the top is porphyrin negative.

include trimethoprim-sulfamethoxazole, imipenem, and ciprofloxacin. Chloramphenicol with ampicillin is also effective. Because of increased resistance to ampicillin, this drug should not be used alone for initial therapy. Non-life-threatening *H. influenzae* infection may be treated with amoxicillin-clavulanate, an oral second-generation or third-generation cephalosporin, or trimethoprim-sulfamethoxazole. For the treatment of *H. ducreyi*, azithromycin, ceftriaxone, ciprofloxacin, or erythromycin is recommended.

Increased resistance to ampicillin by *Haemophilus* spp. resulting from β -lactamase or, to a lesser extent, altered penicillin-binding proteins has been reported. In the United States, approximately 25% of NTHi isolates are β -lactamase positive. Several rapid tests to detect β -lactamase production are available, including the chromogenic cephalosporin test (Cefinase; BD Diagnostic Systems) and acidometric tests (see **Chapter 13**). A positive β -lactamase test means that the microorganism is resistant to ampicillin and amoxicillin.

In the chromogenic cephalosporin test, a disk impregnated with nitrocefin is moistened with a drop of water. Using a sterile loop, several colonies are smeared onto the disk surface, or forceps can be used to wipe the moistened disk across the colonies. If the β -lactam ring of nitrocefin is broken by the enzyme β -lactamase, a red color develops on the area where the culture was applied. The reaction usually occurs within 5 minutes.

In the acidometric test, a strip impregnated with benzylpenicillin and a pH indicator, bromcresol purple, is moistened with one or two drops of sterile distilled water. Using a sterile loop, several colonies are smeared onto the test strip. If the β -lactam ring of the benzylpenicillin is broken by β -lactamase, penicilloic acid is formed, causing a decrease in pH. This decrease in pH is demonstrated by a color change from purple (negative) to yellow (positive) on the strip within 5 to 10 minutes.

Antimicrobial susceptibility testing of *H. influenzae* should be limited only to isolates known to be clinically significant. In most cases, only testing for β -lactamase activity to assess ampicillin and amoxicillin efficacy is necessary. Because of the fastidious nature of the organism, special media and protocols must be followed if antimicrobial susceptibility testing is performed.

Table 18.2 Differential tests for *Haemophilus* and *Aggregatibacter* species

	Oxidase	Catalase	Hemolysis (horse, rabbit blood)	Carbon dioxide enhances growth	ONPG	Glucose	Sucrose	Mannose	Fructose	Mannitol	Maltose	Xylose	Lactose	Nitrate	Esculin	Ornithine	Indole	Urea
Factor X+, V+, porphyrin–																		
<i>H. influenzae</i>	+	+	–	–	–	+	–	–	V	–	–	+	–	+	–	See biotype chart	See biotype chart	See biotype chart
<i>H. haemolyticus</i>	+	+	+	–	–	+	–	–	V	–	–	V	–	+	–	–	V	+
Factor X–, V+, porphyrin+																		
<i>H. parainfluenzae</i>	+	V	–	V	V	+	+	+	+	–	+	–	–	+	–	See biotype chart	See biotype chart	See biotype chart
<i>H. parahaemolyticus</i>	+	+	+	–	–	+	+	–	+	–	+	–	–	+	–	–	–	+
<i>H. paraphrohaemolyticus</i>	+	+	+	+	V	+	+	–	+	–	+	–	–	+	–	–	–	+
<i>A. segnis</i>	–	V	–	–	–	W	W	–	W	–	W	–	–	+	–	See biotype chart		
Factor X+, V–, porphyrin–																		
<i>H. ducreyi</i>	–	–	+/–	+/–	–	V	–	–	–	–	–	–	–	+	–	–	–	–
Factor X–, V–, porphyrin+																		
<i>A. aphrophilus</i> ^a	+/–	–	–	+	+	+	+	V	+	–	+	–	+	+	–	–	–	–

^aOn initial isolation, may appear to be hemin dependent.

+, >90% positive; –, >90% negative; +/–, more positive than negative; ONPG, o-Nitrophenyl-β-D-galactopyranoside; V, variable; W, weak reception.

Table 18.3 Differential tests for *Haemophilus* biogroups

Haemophilus biogroups	Distribution of biotypes						
	Ornithine	Indole	Urea	Meningitis and epiglottitis	Ear infection	Conjunctivitis	Upper respiratory tract
<i>H. influenzae</i>							
Biotype I	+	+	+	••••	••	•	•
Biotype II	–	+	+	•	••	••	•
Biotype III	–	–	+	•	•	••	•
Biotype IV	+	–	+	•	•	•	•
Biotype V	+	+	–	•	•	•	•
Biotype VI	+	–	–	•	•	•	•
Biotype VII	–	+	–	•	•	•	•
Biotype VIII	–	–	–	•	•	•	•
<i>H. parainfluenzae</i>							
Biotype I	+	–	–				
Biotype II	+	–	+				
Biotype III	–	–	+				
Biotype IV	+	+	+				
Biotype V	–	–	–				
Biotype VI	+	+	–				
Biotype VII	–	+	+				
Biotype VIII	–	+	–				

+, >90% positive; –, >90% negative; •, 0% to 25%; ••, 26% to 50%; •••, 51% to 75%; ••••, 76% to 100%.

HACEK group

HACEK is an acronym consisting of the first initial of each genus represented in the group:

Haemophilus spp., e.g., *H. paraphrophilus*

Aggregatibacter actinomycetemcomitans, formerly *Actinobacillus actinomycetemcomitans*, and *A. aphrophilus*, formerly *H. aphrophilus*

Cardiobacterium hominis

Eikenella corrodens

Kingella spp.

Members of this group of gram-negative bacilli have in common fastidious nutritional requirements and enhanced growth with increased CO₂. Members of the genus *Cardiobacterium* are **capnophilic** (require CO₂). In contrast with *Haemophilus* spp., the latter four members of the HACEK group are considered more **dysgonic** (i.e., slower or poorer growing). Their predilection for attachment to heart valves, usually damaged or prosthetic, makes many of them an important cause of endocarditis that often involves the heart valves. The lesion (referred to as **vegetation**) is composed of fibrin, platelets, polymorphonuclear cells, monocytes, and microorganisms. Additional organisms that account for most cases of endocarditis are the viridans group of streptococci (most common after 1 year of age), *S. aureus*, *S. pneumoniae*, coagulase-negative staphylococci, *Abiotrophia* spp., and enterococci.

Members of the HACEK group include both fermentative and nonfermentative, gram-negative bacilli. All members can be normal biota of the oral cavity, which permits their introduction in the bloodstream and resultant infections. All

HACEK organisms are opportunists and generally require a compromised host. Risk factors for infective (bacterial) endocarditis include tooth extraction, history of endocarditis, gingival surgery, heart valve surgery, and mitral valve prolapse. Table 18.4 summarizes the key reactions and characteristics of HACEK and *Capnocytophaga* spp.

Aggregatibacter aphrophilus

A. aphrophilus (Greek *aphros* and *philia*: foam loving or desiring high concentration of CO₂) is one of the most prevalent species in the HACEK group involved in endocarditis. This organism has also been linked to bone and joint infections. Although it does not require CO₂, it does grow better in its presence. *A. aphrophilus* is found in dental plaque and gingival scrapings. Patients with infections present commonly with clinical features of fever, heart murmur, congestive heart failure, and embolism. *H. aphrophilus* and *H. paraphrophilus* have been reclassified into the single species, *A. aphrophilus*. This species contains V factor–dependent (see Fig. 18.7) and V factor–independent strains (Fig. 18.10). *A. aphrophilus* colonies are convex, granular, and yellow with an opaque zone near the center on CHOC agar. Figs. 18.11 and 18.12 illustrate the colony and microscopic morphology of *A. aphrophilus*.

Aggregatibacter actinomycetemcomitans

A. actinomycetemcomitans was formerly in the genus *Actinobacillus*. The remaining members of *Actinobacillus* include animal pathogens or animal endogenous biota that in general do not routinely cause human infections. Human

Table 18.4 Summary of key reactions and characteristics of HACEK and *Capnocytophaga* species

	Catalase	Oxidase	Glucose	Maltose	Sucrose	Lactose	Comments
<i>Aggregatibacter aphrophilus</i> Gram stain: small coccobacillus Colony morphology: raised, convex, granular, yellowish	–	V	+	+	+	+	
<i>Aggregatibacter actinomycetemcomitans</i> Gram stain: very small coccobacillus Colony morphology: small colonies that adhere to agar	+	V	+	V	–	–	
<i>Cardiobacterium hominis</i> Gram stain: straight bacilli, spindles, rosettes Colony morphology: smooth, opaque, adherent to agar	–	+	+	+	+	–	Indole +
<i>Eikenella corrodens</i> Gram stain: straight bacilli Colony morphology: usually pits the agar	–	+	–	–	–	–	Smells like bleach Ornithine +
<i>Kingella kingae</i> Gram stain: coccoid to straight bacilli, chains and pairs, square ends Colony morphology: two types: spreading and corroding or smooth and convex β -hemolysis under colony	–	+	+	+	–	–	Nitrate–
<i>Capnocytophaga</i> spp. Gram stain: long, thin bacilli; tapered ends Colony morphology: flat colonies, irregular in shape, may appear purple	– ^a	– ^a	+	+	+	+	Indole –

+, Positive; –, negative; V, variable.
^a*C. canimorsus* and *C. cynodegmi* are catalase and oxidase positive.

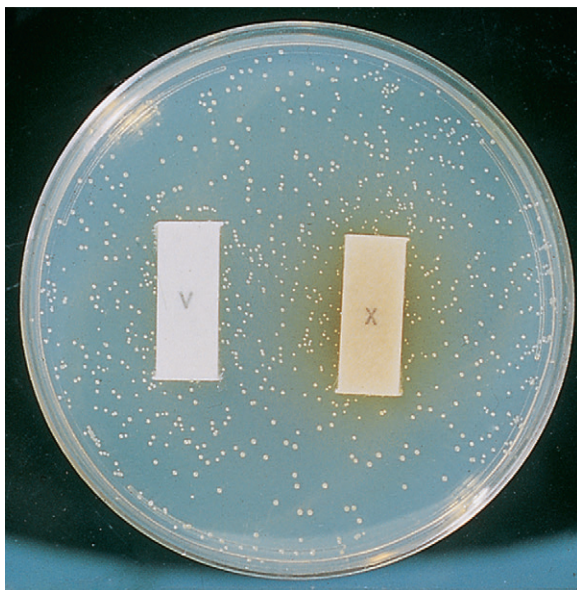


Fig. 18.10 An *Aggregatibacter aphrophilus* isolate that is not X factor dependent and is growing over the entire surface of a trypticase soy agar plate.

tissue infections have been attributed to bites by cattle, sheep, pigs, and horses or through contact with these animals. *A. actinomycetemcomitans* is divided into six serotypes (a to f) based on a surface polysaccharide, of which a, b, and c are the most common. The organism is found as normal oral microbiota in humans and is a major contributor to periodontitis (Fig. 18.13). Individuals with juvenile periodontal disease or other dental disease harbor the organism, and it can cause destruction of the alveolar bone that supports teeth in these individuals. *A. actinomycetemcomitans* has been isolated from blood, lung tissue, abscesses of the mouth and brain, and sinuses. It has been linked to subacute bacterial endocarditis with an insidious and protracted presentation. Major virulence factors include collagenase and a leukotoxin that is toxic to polymorphonuclear cells and monocytes.

A. actinomycetemcomitans grows better with increased CO₂. The isolates may require more than 24 hours for visible growth; a distinctive “star shape with four to six points” in the center of the colonies is often seen at 48 hours. The star shape is best observed after 48 hours by using $\times 100$ magnification under a light microscope when grown on a clear medium or a stereomicroscope at the highest magnification available. Fig. 18.14 depicts the colony morphology. In broth,



Fig. 18.11 *Aggregatibacter aphrophilus* growing on sheep blood agar.

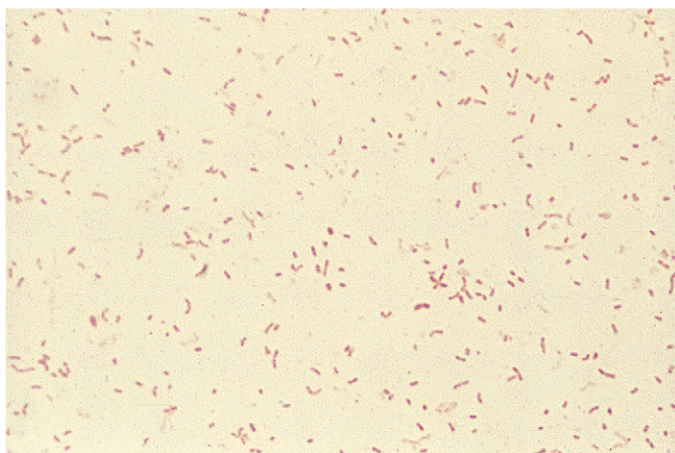


Fig. 18.12 Gram stain morphology of *Aggregatibacter aphrophilus* ($\times 1000$).



Fig. 18.13 Advanced periodontitis. Periodontitis is inflammation of the periodontium caused by a complex reaction initiated when subgingival plaque bacteria are in close contact with the epithelium of the gingival sulcus. (From Murray, P. R., et al. [1994]. *Medical microbiology* [2nd ed.]. St. Louis: Mosby.)



Fig. 18.14 *Aggregatibacter actinomycetemcomitans* on sheep blood agar. The star-shaped centers of the colonies are not usually evident until after 48 hours of incubation and are best observed by using $\times 100$ magnification (light microscope) or a stereomicroscope.

the organism is granular and may adhere to the sides of the tube. Isolates are catalase positive and oxidase variable, do not grow on MAC agar, and are negative for X and V growth factor requirements, urease, indole, esculin, and citrate. *A. actinomycetemcomitans* is typically urease negative, which differentiates it from the members of the genus *Actinobacillus*. Glucose fermentation is positive (with or without gas), although the addition of serum to the carbohydrate-containing medium is often necessary to demonstrate fermentation. Xylose, mannitol, and maltose fermentation are variable. The isolates do not ferment lactose or sucrose.

A. actinomycetemcomitans demonstrates sensitivity to penicillin in vitro, although this agent is not always successful clinically, and resistance to ampicillin is common. Isolates are typically susceptible to aminoglycosides, third-generation cephalosporins, quinolones, chloramphenicol, and tetracycline. Resistance to vancomycin and erythromycin is common. Usual treatment for endocarditis is with penicillin and an aminoglycoside.

Cardiobacterium hominis

The genus *Cardiobacterium* contains two species: *C. hominis* and *C. valvarum*. Both are pleomorphic, nonmotile, fastidious, gram-negative bacilli found as normal microbiota of the nose, mouth, and throat and may be present in the gastrointestinal tract. Oral infections or dental procedures usually precede endocarditis. The usual clinical manifestation is endocarditis, often manifesting with very large vegetations and no demonstrable fever. It infects the aortic valve more frequently compared with the other HACEK organisms. Rarely, *C. hominis* has been associated with meningitis.

Gram-stained smears of the bacilli often show false gram-positive reactions in parts of the cells. The organisms tend to form rosettes, swellings, long filaments, or sticklike structures in yeast extract. They grow slowly on SBA and CHOC agar and fail to grow on MAC agar. Incubation in a humid atmosphere (either aerobic or anaerobic) with 5% CO₂ is required for growth. Figs. 18.15 and 18.16 illustrate the colony and microscopic morphology. On agar, "pitting" can be produced. *C. hominis* is a fermenter, but as with *A. actinomycetemcomitans*, reactions may be weak, and serum might be needed. Isolates are oxidase positive, catalase negative, and indole positive; the latter two traits help differentiate them from *Aggregatibacter* spp. *C. hominis* is negative for



Fig. 18.15 Growth of colonies of *Cardiobacterium hominis* over 48 hours on sheep blood agar.

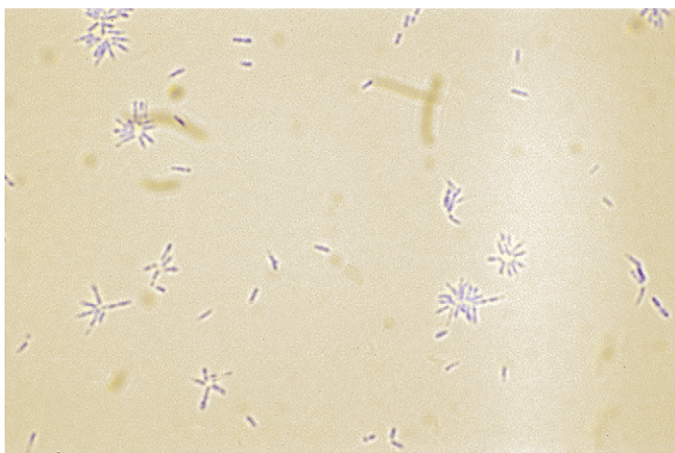


Fig. 18.16 Gram-stained smear of *Cardiobacterium hominis* showing typical "rosettes" (×1000).

urease, nitrate, gelatin, and esculin. Sensitivity can be seen to β -lactams, chloramphenicol, and tetracycline with variable response to aminoglycosides, erythromycin, clindamycin, and vancomycin. Usual therapy includes penicillin and an aminoglycoside.

Eikenella corrodens

E. corrodens is a member of the normal biota of the oral and bowel cavities. Most infections associated with this organism have been polymicrobial and often occur as a result of trauma, especially after human bites or fights (i.e., "clenched fist wounds," or after the skin has been broken by human teeth). Poor dental hygiene or oral surgery has also been associated with infections. It is an opportunistic pathogen, especially in immunocompromised individuals. *E. corrodens* has been reported as the cause of adult periodontitis, meningitis, empyema, pneumonia, osteomyelitis, arthritis, and postoperative tissue infections. In those with drug addiction, it has been implicated in cellulitis as a result of direct inoculation of the organisms into the skin after oral contamination of needle paraphernalia (because of licking the needle for cleaning, instead of sterilizing, and for good luck). *E. corrodens* shows a predilection for attachment to heart valves and

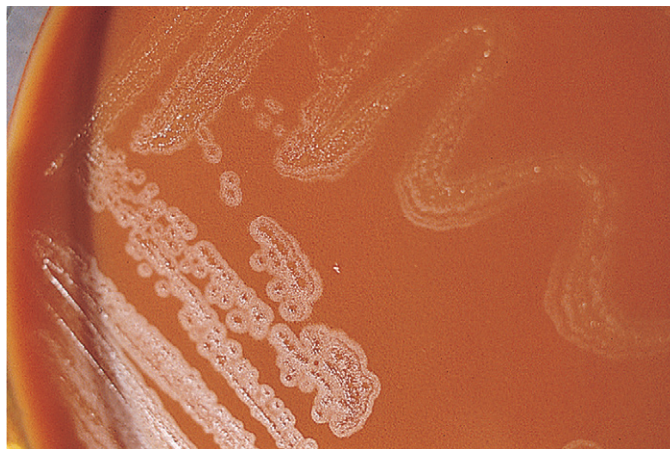


Fig. 18.17 Growth of *Eikenella corrodens* on chocolate agar. (Compare this growth with *Capnocytophaga* in Fig. 18.20.)

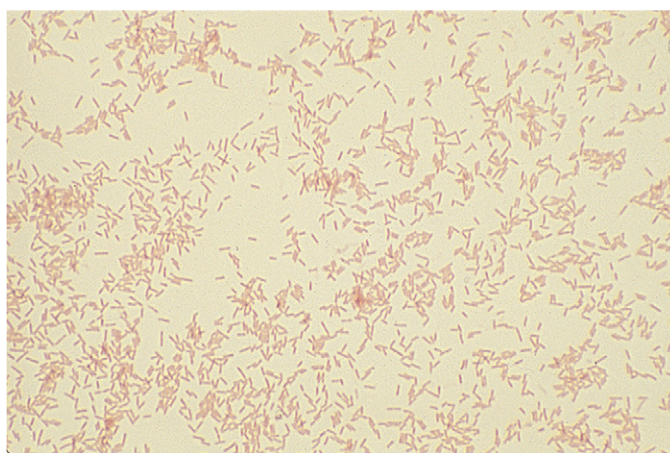


Fig. 18.18 Gram stain morphology of *Eikenella corrodens* (×1000).

causes endocarditis, although it is probably the least common isolate of the HACEK group in adult infectious endocarditis.

E. corrodens isolates are fastidious, gram-negative coccobacilli that grow best under conditions of increased CO₂ with hemin. They are nonmotile, oxidase positive, and asaccharolytic. They are catalase negative and often produce a yellow pigment. About 45% of the isolates of *E. corrodens* "pit" (make a depression) or corrode the surface of the agar. Figs. 18.17 and 18.18 illustrate the colony and microscopic morphology. Although they are nonhemolytic on SBA, a slight greening effect secondary to growth may occur around the colonies. A chlorine bleach-like odor from the agar surface may be obvious. Isolates do not usually grow on MAC agar or eosin-methylene blue (EMB) agar. In broth media, *E. corrodens* may adhere to the sides of the tube and produce granules. Typically, *E. corrodens* is resistant to clindamycin and narrow-spectrum cephalosporins. In vitro, isolates demonstrate sensitivity to penicillin, ampicillin, cefoxitin, chloramphenicol, carbenicillin, and imipenem.

Kingella

The genus consists of six species: *Kingella kingae*, *K. denitrificans*, *K. negevensis*, *K. oralis*, *K. bonacorsii*, and *K. potus*. *Kingella*

spp., especially *K. kingae*, are recognized as important pathogens in the pediatric population and have a predilection for bones and joints. It appears to be the most common cause of osteoarthritis infection in children younger than 4 years of age. *K. kingae*, a member of the normal oropharynx microbiota, has also been associated with endocarditis, particularly in immunocompromised patients. Isolates have been obtained clinically from blood, bone, joint fluid, urine, and wounds. *K. denitrificans* has been associated with endocarditis. Poor dental hygiene or oral surgery is associated with infection by *Kingella* spp.

Members of the genus *Kingella* are coccobacillary to short bacilli with squared ends that occur in pairs or short chains (Fig. 18.19). They tend to resist decolorization on Gram stain. *Kingella* spp. are typically nonmotile. They are nutritionally fastidious, oxidase-positive, catalase-negative, and ferment glucose and other sugars but with no gas.

Kingella spp. can grow on *Neisseria* selective agar (e.g., modified Thayer-Martin [MTM] medium) and can resemble *Neisseria gonorrhoeae* if the isolate does not pit the agar as many strains do. *K. denitrificans*, like *N. gonorrhoeae*, produces acid from glucose. Gram-stain morphology of a rod with square ends and in chains and a negative catalase test result aid in distinguishing *Kingella* spp. from *N. gonorrhoeae*. Isolates with the following characteristics meet the CLSI abbreviated test requirements for *K. kingae*: gram-negative, short, coccoid bacilli forming large white-to-beige β -hemolytic colonies on SBA, no growth on MAC agar, catalase negativity, and oxidase positivity. *K. denitrificans* is positive for glucose fermentation and nitrate reduction and might grow at 42° C. *K. denitrificans* has two types of colonies: a smooth, convex type and a spreading, corroding type.

K. kingae weakly ferments glucose and maltose but is negative for sucrose. In contrast with other *Kingella* spp., it may produce a yellow-brown pigment. *K. kingae* has two types of colony morphologies: a spreading, corroding colony or a smooth, convex, and β -hemolytic colony. The hemolysis may appear beneath the colony or in close proximity after 24 hours. After 48 hours of incubation, the hemolysis is more evident. Isolates of *Kingella* spp. are usually susceptible to most agents, including penicillin.



Fig. 18.19 Gram-stained smear of *Kingella kingae* illustrating the plump bacilli in chains and tendency to resist decolorization. Compare with other members of the HACEK group ($\times 1000$).

Capnocytophaga

Capnocytophaga belongs to the family Flavobacteriaceae and includes bacteria previously called DF-1 and DF-2 (dysgonic fermenters). The genus consists of at least 14 species, 6 of which are normal microbiota of the oral cavity of humans and have been associated with septicemias and other human infections. *Capnocytophaga* spp. are not as commonly involved in endocarditis as they are in septicemia, notably in patients with neutropenia. Common sites of clinical isolation include blood cultures from patients who have neutropenia with oral ulcers (source of the *Capnocytophaga*), soft tissue infections, peritonitis, and endocarditis. *C. ochracea* is the most common clinical isolate. *C. canimorsus* and *C. cynodegmi* are normal inhabitants of the oral cavity of dogs and cats. *C. canimorsus* can cause a fulminant, life-threatening septicemia in humans, particularly in patients with asplenia or alcoholism, after a dog or cat bite, or through continuous contact. This etiologic agent should be considered in patients who have a history of dog bites or saliva transfer and progressive illness.

Capnocytophaga spp. are fastidious, facultatively anaerobic, gram-negative bacilli and require increased CO₂ for growth and isolation from blood cultures. They are thin and often fusiform (pointed ends) resembling *Fusobacterium* spp.; spindle-shaped, coccoid, and curved filaments may be also seen. Although flagella are usually absent, *Capnocytophaga* can produce gliding motility on solid surfaces. On agar, colonies are often adherent and produce a yellow-orange pigment; they can resemble colonies of *E. corrodens*. Most *Capnocytophaga* isolates are nonhemolytic except for *C. haemolytica* (β -hemolytic). Figs. 18.20 and 18.21 depict the colony and microscopic morphology.

Capnocytophaga spp. ferment sucrose, glucose, maltose, and lactose, although triple sugar iron agar (TSIA) may be negative without enrichment. These organisms are negative for most biochemical reactions, including indole. The six normal inhabitants of the human oral cavity—*C. ochracea*, *C. gingivalis*, *C. sputigena*, *C. haemolyticus*, *C. leadbetteri*, and *C. granulosa*—are all oxidase and catalase negative. *C. canimorsus* and *C. cynodegmi* are oxidase and catalase positive. *Capnocytophaga* spp. are susceptible to imipenem, erythromycin, clindamycin,



Fig. 18.20 Growth of *Capnocytophaga* organisms on chocolate agar. Note the spreading away from the center of the colony. (Compare this growth with *Eikenella* in Fig. 18.17).

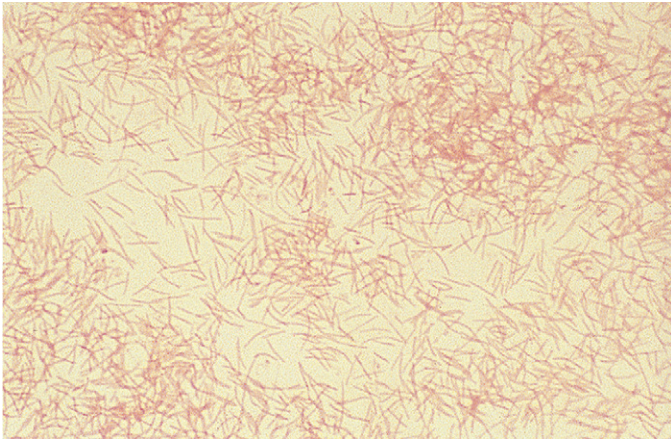


Fig. 18.21 Gram stain morphology of *Capnocytophaga* sp. (×1000). Note the thin fusiform bacilli.

tetracycline, chloramphenicol, quinolones, and β -lactams, but they are resistant to the aminoglycosides. For *C. canimorsus* and *C. cynodegmi* infections, penicillin is the drug of choice.

Pasteurella

Pasteurellosis, infection with *Pasteurella* spp., is a **zoonosis**—a disease that humans acquire from exposure to infected animals or products made from infected animals. Although systemic (septicemia, arthritis, endocarditis, osteomyelitis, meningitis) and pneumonic forms are possible, soft tissue (cutaneous) infection, frequently resulting from animal bites, is the most common presentation. These wounds can become infected with *Pasteurella* spp. because these organisms often reside in the respiratory tracts and oral cavities of birds and mammals. Cutaneous infections can quickly progress, resulting in inflammation and exudate production. Although *Pasteurella* infections can result from a variety of animal bites, they often occur as the result of feline bites.

At least 17 species of *Pasteurella* have been identified; based on deoxyribonucleic acid (DNA) hybridization, several species are more closely related to other genera, such as *Actinobacillus*. *Pasteurella multocida* is the most frequently isolated species and includes four subspecies: *multocida*, *septica*, *tigris*, and *gallicida*. *P. multocida* also consists of five serogroups (A, B, D, E, and F) defined by capsular antigens. *P. canis*, associated with dogs, and *P. stomatis* and *P. dagmatis*, both associated with dogs and cats, are also isolated infrequently from humans.

Pasteurella spp. are gram-negative, nonmotile, facultative, anaerobic coccobacilli that appear ovoid, filamentous, or as bacilli. **Bipolar staining** (safety pin appearance when the poles of the cells are more intensely stained) is frequently observed. These bacteria are catalase and oxidase (most isolates) positive and ferment glucose with weak to moderate acid production without gas.

All *Pasteurella* spp. grow on SBA and CHOC agar, producing grayish colonies (Fig. 18.22). Conversely, MAC agar does not support the growth of most *Pasteurella* spp. Growth on SBA in the absence of satellitism or in pure culture combined with bipolar staining may differentiate *Pasteurella* from *Haemophilus*. *P. multocida* produces nonhemolytic colonies on SBA that may appear mucoid after 24 hours of incubation at 35° C followed by the production of a narrow green-to-brown

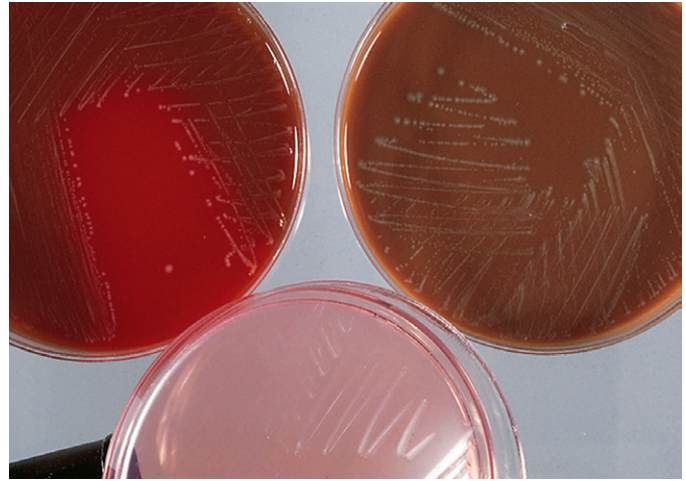


Fig. 18.22 *Pasteurella multocida* growing on sheep blood agar and chocolate agar. The MacConkey agar plate is negative for growth.

halo around the colony after 48 hours. Table 18.5 lists the various growth characteristics and biochemical reactions that can be useful in the differentiation of *Pasteurella* spp. associated with human infections.

Brucella

Brucellosis, infection with bacteria from the genus *Brucella*, is an important zoonotic disease that is found throughout the world. There are eight named terrestrial species. However, on the basis of genetic similarity, it has been proposed that there be a single species with six biovars. The most prevalent species reported in terrestrial wildlife is *B. abortus*. The four species that are most commonly associated with human illness are *B. melitensis*, *B. abortus*, *B. suis*, and *B. canis*. The four other species are *B. ovis*, *B. neotomae*, *B. microti*, and *B. papionis*. In recent years, additional species have been isolated from marine mammals. Because of their potential application in bioterrorism, *Brucella* spp. are considered category B **select biological agents** by the CDC. Category B agents are easy to disseminate and cause moderate morbidity but low mortality. U.S. Federal Code 42CFR72.6 describes the requirements for possession and transfer of select biological agents. For more information about biological threat agents, see Chapter 30. Brucellosis is a CDC-reportable disease because the diagnosis can have public health implications. Approximately 100 cases are reported annually.

Brucellosis has been described by both the disease course (undulant fever) and the geographic locations where cases have occurred (Mediterranean, Crimean, and Malta fevers). Certain subpopulations are at higher risk of contracting brucellosis, such as individuals exposed to animals and animal products (e.g., veterinarians, hunters) and laboratory workers. Brucellosis is acquired through aerosol, percutaneous, and oral routes of exposure. Oral exposure can occur by aerosol or ingestion of unpasteurized milk. Although direct person-to-person transmission is considered rare, cases resulting from sexual contact and breast-feeding have been reported.

The resulting clinical presentation appears to be similar for all exposure routes. The three clinical stages of brucellosis, based arbitrarily on disease duration, are the acute, subchronic, and chronic stages. Symptoms of acute infection

Table 18.5 Differential characteristics of *Pasteurella* species associated with human infections

Species/ subspecies	Normal habitat	Hemolysis	Oxidase	Catalase	ODC	Indole	Growth on MacConkey	Urease	Comments
<i>P. multocida</i>	Oral cavities of healthy domestic dogs, cats, and other animals (swine, horses, cattle)	–	+	+	+	+	–	–	Most common isolate from human specimens; most infections are associated with dog and cat bites and scratches; can cause respiratory tract infections, including lung abscesses, pneumonia, empyema, and tonsillitis; usually underlying disease present or immune complications; differentiated by acid production from dulcitol and sorbitol; rare clinical isolates, primarily of veterinary interest
<i>P. pneumotropica</i>	Respiratory tract of dogs, cats, and some rodents	–	+	+	+	+	V	+	Human infections acquired mostly through dog and cat bites
<i>P. dagmatis</i>	Respiratory tract of dogs and cats	–	+	+	–	+	–	+	Human infections caused by bites and scratches of dogs and cats
<i>P. stomatis</i>	Oropharyngeal biota of dogs and cats	–	+	+	–	+	–	–	Caused by bites
<i>P. canis</i>	Biotype I is found in the oral cavity of dogs	–	+	+	+	+	–	–	Wound infections caused by dog bites
<i>P. bettyae</i>	Isolated from genitourinary tract including vagina, cervix, Bartholin glands, and amniotic fluid	–	V	–	–	+	V	–	Pathogen may be sexually transmitted; formally known as CDC group HB-5

+, Greater than 90% positive; –, greater than 90% negative; CDC, Centers for Disease Control and Prevention; ODC, ornithine decarboxylase; V, 20% to 80% positive.

are nonspecific (fever, malaise, headache, anorexia, arthralgia, myalgia, and back pain) and usually occur within 1 to 4 weeks of exposure. Brucellosis is a systemic, deep-seated disease resulting in various long-term sequelae. Mortality is low and generally results from cardiac disease. The subchronic or undulant fever form of the disease typically occurs within 1 year of exposure and is characterized by undulating fevers (characterized by body temperatures that rise in the afternoon and evening and fall in the morning), arthritis, and epididymo-orchitis (inflammation of the epididymis and testis). The chronic form commonly manifests 1 year after exposure with symptoms such as depression, arthritis, and chronic fatigue syndrome.

Brucellae are small gram-negative, aerobic, nonmotile, unencapsulated bacteria that may appear as coccobacilli or bacilli. Under optimal growth conditions on agar, smooth, raised, and translucent colonies appear (Fig. 18.23). The bacteria are facultative intracellular pathogens that can reside within mononuclear phagocytic cells. It can be difficult to diagnose brucellosis through direct examination of a clinical sample, most often blood or bone marrow, and the ability for direct isolation and culture can vary between acute and

chronic manifestations. The bacteria are sometimes recovered from CSF, synovial fluid, pleural fluid, and abscesses. Of acute cases, 50% to 80% yield positive blood cultures, whereas only 5% of chronic cases produce positive cultures. As a result, serologic tests are frequently used, in conjunction with patient history and disease status, to diagnose brucellosis.

Brucella spp. can vary in their requirement for CO₂. They grow on SBA and CHOC agar and can be isolated on MTM or Martin-Lewis media from contaminated specimens. Growth usually appears within 24 to 48 hours, and plates should be held for 4 days before being reported as negative. Brucellae are oxidase and catalase positive and are urease positive within 2 hours (Table 18.6). These tests can also differentiate brucellae from similar organisms (e.g., *Bordetella bronchiseptica*, *Acinetobacter* spp., and *H. influenzae*) when combined with growth on SBA, colony morphology, specimen source, and testing for X factor and V factor requirements (Table 18.7). MALDI-TOF mass spectrometry can be used for identification of *Brucella* species if the organism is included in the database being used (e.g. Bruker's biosecurity-relevant library). Members of *Brucellae* have been commonly

misidentified as *Oligella ureolytica*, *Psychrobacter phenylpyruvica*, and *Ochrobactrum anthropi* on MALDI-TOF mass spectrometry libraries that do not include *Brucella* species. *Brucella* species can be identified by real-time PCR and subtyped into biovars using molecular biology techniques.



Fig. 18.23 *Brucella melitensis* colonies on sheep blood agar appear smooth, raised, and translucent. (From the CDC Public Health Image Library. <http://www.phil.cdc.gov/phil>. Courtesy Larry Stauffer, Oregon State Public Health Laboratory.)

Because of the aerosol mode of transmission, *Brucella* spp. should be handled under biosafety level 3 conditions (e.g., within a biological safety cabinet). Approximately 2% of all reported cases of brucellosis are acquired in the laboratory, illustrating the risk of infection to clinical laboratory personnel. The chance of acquiring an infection from a laboratory exposure ranges from 30% to 100% and depends on a range of factors.

Francisella

According to the current taxonomic classification, there are about 14 species in the genus *Francisella*. Although *F. tularensis* has been implicated in most human infections, *F. novicida* and *F. philomiragia* have also been known to infect patients with immune-compromising conditions. *F. tularensis* has four subspecies *tularensis* (type A), *holarctica* (type B), *novicida*, and *mediasiatica*. *F. tularensis* subsp. *tularensis* causes the most severe disease; *F. tularensis* subsp. *holarctica* produces a disease similar to that by *F. tularensis* subsp. *tularensis*, but infections are rarely fatal.

Tularemia, which is infection with bacteria from the genus *Francisella*, is a zoonotic disease and has many other names, including *rabbit fever*, *deerfly fever*, *lemming fever*, and *water rat trappers' disease*. Tularemia can be contracted through ingestion, inhalation, arthropod bite (e.g., ticks, biting flies), or contact with infected tissues. Clinical presentation can assume various forms and is influenced by the route of bacteria

Table 18.6 Differential characteristics of *Brucella* species associated with human infections

<i>Brucella</i> species	Natural hosts	Serum agglutination (patient antibodies)	H ₂ S (lead acetate)	Urease	CO ₂ (enhanced growth)	Inhibition of growth in dyes	
						Thionine	Fuchsin
<i>B. melitensis</i>	Goat or sheep	+	–	V	–	–	–
<i>B. abortus</i>	Cattle	+	+	+<2 hours	+/-	+	–
<i>B. suis</i>	Swine	+	+	+<0.5 hour	–	–	+
<i>B. canis</i>	Dogs	–	–	+<0.5 hour	–	–	+

+, >90% positive; –, >90% negative; +/-, more positive than negative; CO₂, Carbon dioxide; H₂S, hydrogen sulfide; V, variable.

Table 18.7 Differential characteristics for the identification of *Brucella* from similar organisms

Test	<i>Brucella</i> spp.	<i>Bordetella bronchiseptica</i>	<i>Acinetobacter</i> spp.	<i>Psychrobacter phenylpyruvica</i> ^a	<i>Oligella ureolytica</i>	<i>Haemophilus influenzae</i>
Oxidase	+ ^b	–	–	+	+	V
Motility	–	+	–	–	V	–
Urea hydrolysis	+	+	V	+	+	V
Nitrate reduction	+	+	–	V	+	+
Growth SBA	+	+	+	+	+	–
Cellular morphology	Tiny ccb stains faint	Small ccb, bacilli	Broad ccb	Broad ccb	Tiny ccb	Small ccb
Specimen source	Blood, bone marrow	Respiratory tract	Various sites	Various sites	Urinary tract	Mucous membranes
X or V factor requirement	–	–	–	–	–	+

^aFormerly *Moraxella phenylpyruvica*.

^b*B. abortus*, *B. melitensis*, and *B. suis* are 95% positive; *B. canis* is 72% positive.

+, >90% positive; –, >90% negative; V, variable (11% to 89% positive); ccb, coccobacillus; SBA, sheep blood agar.

From Centers for Disease Control and Prevention. Presumptive *Brucella* spp. identification and similar organisms. Available at: <https://emergency.cdc.gov/documents/pptresponse/table5brucellaaid.pdf>.

exposure. The most common clinical form, reported in 45% to 80% of cases, is ulceroglandular, in which an ulcer forms at the site of inoculation and is followed by an enlargement of the regional lymph nodes. Tularemia can also occur in pneumonic (contracted via the inhalation route), glandular, oropharyngeal, oculoglandular, and typhoidal forms. Case fatality rates for tularemia in the United States range from 2.3% to 9%. *F. tularensis* is a CDC category A select biological agent. Category A agents are described as posing a risk to national security because they can be spread through person-to-person contact or are easily disseminated and result in high mortality rates, leading to a potentially great public health impact and public panic. Similar to brucellosis, tularemia is a CDC-reportable disease, and about 100- to 200 cases are reported annually.

The preferred specimen for isolation of *Francisella* spp. depends on clinical manifestations. Blood is an acceptable specimen for all forms; however, cultures can be negative early in the course of the infection. *Francisella* spp. appear as small, nonmotile, gram-negative bacilli or coccoid bacteria and are strictly aerobic. Similar to *Brucella* spp., *Francisella* are also facultative intracellular parasites. *Francisella* spp. are fastidious and require supplementation with cysteine, cystine, or thiosulfate for growth on successive passage. CHOC, MTM, and buffered charcoal yeast extract (BCYE) agars and thioglycollate broth may be used. However, because of the risks of laboratory infections from aerosols, broth cultures are not recommended. MAC and EMB agars do not support *F. tularensis* growth. Because of slow growth rates, *F. tularensis* colonies may not be visible before 48 hours of incubation at 35° C. Plates should be checked daily for 14 days. Once visible, gray-white, raised colonies with a smooth appearance are seen (Fig. 18.24).

F. tularensis is oxidase, urease, and satellite or X and V test negative and weakly positive for catalase and β -lactamase activity. Additionally, clinical symptoms, patient exposure in geographic locations where tularemia is endemic, and

serology are used to diagnose *F. tularensis* infection. A presumptive identification can be made by a positive test result in any one of the following assays: direct fluorescent antibody (DFA), immunohistologic staining with monoclonal antibody, PCR, slide agglutination, or single serology test. Confirmation requires culture identification or a fourfold increase in antibody titer. MALDI-TOF mass spectrometry has been shown to be an accurate method to identify *Francisella* strains, including the subspecies of *F. tularensis*.

F. tularensis is a highly infectious agent with as few as 50 organisms causing an infection through the cutaneous (ulceroglandular) or inhalation (pneumonic) routes and has been the cause of many laboratory-acquired infections. Three Boston University scientists were accidentally infected while working with a fully virulent strain. Laboratory personnel should use appropriate laboratory safety techniques and precautions. Biosafety level 3 conditions should be implemented when working with suspected *F. tularensis* samples.

Legionella

In 1976 during an American Legion convention in Philadelphia, 221 persons developed pneumonia, and 34 of them died of a mysterious disease. *Legionella pneumophila*, the causative agent of this outbreak, became the first named member of the family Legionellaceae, which now contains three genera: *Legionella*, *Fluoribacter*, and *Tatlockia*. The genus *Legionella* includes over 60 species and more than 70 serogroups. The primary human pathogen, *L. pneumophila*, contains 15 serogroups. Almost one half of the named species have been isolated from human clinical specimens.

The identification of *Legionella* from patient specimens can be achieved in most microbiology laboratories with the use of commercial media and other diagnostic methods. An understanding of the microscopic and colony morphology of the *Legionella* spp., their nutritional requirements, the pathogenesis, and the capabilities of various commercial laboratory tests enables clinical microbiologists to reliably identify these microorganisms.

General characteristics

Approximately 26 species of *Legionella* have been isolated from humans. They are ubiquitous gram-negative bacilli acquired primarily through inhalation from environmental sources. Many species can survive inside free-living amoebae. Infected patients can present with a wide variety of conditions. The laboratory diagnosis of *Legionella* infection typically depends on one or more of the following methods:

- Isolation using special media
- MALDI-TOF mass spectrometry
- Urine antigen detection
- Fluorescent antibody staining
- Nucleic acid amplification (e.g., PCR)
- Serology

Virulence factors

The most significant virulence factor of the legionellae is the ability to exist as intracellular pathogens, in amoebae and



Fig. 18.24 *Francisella tularensis* colonies grown on chocolate agar. After 72 hours of incubation, gray-white, raised colonies with a smooth appearance are visible. (From the CDC Public Health Image Library. <http://www.phil.cdc.gov/phil>. Courtesy Larry Stauffer, Oregon State Public Health Laboratory.)

mammalian cells. The bacteria survive inside phagosomes and prevent phagolysosome formation. *Legionella* spp. cause intracellular infections in humans and spread cell to cell. Differing presentations of *Legionella* spp. infection may be influenced by several factors, including the organism's ability to enter, survive, and multiply within the host's cells, especially bronchoalveolar macrophages, and the ability to produce proteolytic enzymes.

Infections caused by *Legionella*

Infections caused by *Legionella* spp. produce a spectrum of symptoms ranging from acute febrile disease with pneumonia (**Legionnaires' disease**), influenza-like febrile disease (**Pontiac fever**), and asymptomatic infection. The mode of transmission and the number of infecting organisms in the inoculum play a role in the clinical features of the infection. In addition, host factors, such as a suppressed immune system, chronic lung disease, alcoholism, and heavy smoking, predispose individuals to Legionnaires' disease. *Legionella* spp. are transmitted to human hosts from environmental sources, primarily via aerosolized water particles or gardening materials in the case of soil-associated *L. longbeachae*. Transmission between humans has not been demonstrated.

Legionnaires' disease

Legionnaires' disease typically manifests in three major patterns: (1) sporadic cases, which are most common and usually occur in the community; (2) epidemic outbreaks, characterized by short duration and low attack rates; and (3) healthcare-associated clusters, occurring in compromised patient populations. Pneumonia is the predominant manifestation of Legionnaires' disease, and the organisms are among the top four causes of community-acquired bacterial pneumonia. *S. pneumoniae* is the most common cause of bacterial pneumonia. *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae*, and *Legionella* produce symptoms different from *S. pneumoniae*, causing a disease sometimes referred to as **atypical pneumonia**. The mortality rate for Legionnaires' disease is 15% to 30% and may approach 50% in patients with healthcare-associated pneumonia if the correct diagnosis is not made early.

L. pneumophila serogroup 1 accounts for most cases of Legionnaires' disease, approximately 95%. The incubation period is 2 to 14 days, with a median of about 4 days, and patients typically present with a nonproductive cough, fever, headache, and myalgia. Later, as pulmonary infiltrates develop, sputum may be bloody or purulent. Rales, dyspnea, and shaking chills are clinical manifestations of progressing disease. Dissemination via the circulatory system can lead to extrapulmonary infections with or without pneumonia. Infections of the kidneys, liver, heart, CNS, lymph nodes, spleen, and bone marrow and cutaneous abscesses have been described. Bacteremia, renal failure, liver function abnormalities, watery diarrhea, nausea, vomiting, headache, confusion, lethargy, and other CNS abnormalities have been associated with these infections.

Pontiac fever

The nonpneumonic form of legionellosis, Pontiac fever, usually has a short incubation period of about 2 days. Patients

are previously healthy individuals who complain of flulike symptoms of fever, headache, and myalgia that last 2 to 5 days and then subside without medical intervention. No deaths have been reported. Studies suggest that the disease might be caused by inhalation of a bacterial toxin, such as endotoxin, or an acute allergic reaction to a bacterium. It is unclear if *Legionella* spp. play a role in this condition.

Epidemiology

Approximately 5000 cases of legionellosis are reported annually in the United States; however, many cases likely go undiagnosed. Physicians might not suspect *Legionella* spp., and many laboratories do not routinely culture for these agents. The CDC estimates an incidence of 18,000, and the number of cases has been increasing since 2000. *Legionella* spp. are responsible for 2% to 15% of community-acquired pneumonias in the United States annually. These microorganisms are also associated with healthcare-associated infections, often linked to contaminated equipment and water lines.

Members of the family Legionellaceae are found worldwide, occurring naturally in aquatic sources, such as lakes, rivers, hot springs, and mud. *L. longbeachae* is notable because human infections are associated with exposure to gardening materials, such as compost and potting soil, and constitutes a significant source of infection in New Zealand and Australia. Some species have been recovered only from environmental sources. Because *Legionella* spp. can tolerate chlorine concentrations of 3mg/L, they resist water treatment and subsequently gain entry into and colonize human-made water supplies and distribution systems. Hot water systems, cooling towers, and evaporative condensers are major reservoirs. Other sources include cold water systems, ornamental fountains, whirlpool spas, humidifiers, respiratory therapy equipment, and industrial process water. The factors that contribute to the ability of *Legionella* spp. to colonize these sources include:

- The ability to multiply over the temperature range of 20° to 43° C and survive for varying periods at 40° to 60° C
- The capacity to adhere to pipes, rubber, plastics, and sediment and persist in piped water systems, even when flushed
- The ability to survive and multiply within free-living protozoa and in the presence of commensal bacteria and algae

Laboratory diagnosis

Several methods, such as direct examination, culture, and antigen and antibody detection, are available for the laboratory diagnosis of infections caused by *Legionella* spp. Legionnaires' disease is best diagnosed using a combination of culture and urine antigen detection. Most laboratories use more than one method to maximize their diagnostic capabilities. Because of low sensitivity and false-positive test results from cross-reaction with other bacteria, DFA testing (see Fig. 18.28, later) is no longer recommended. A multiplex PCR pneumonia panel (BioFire FilmArray) that includes *L. pneumophila* along with 32 other targets has been approved by the U.S. Food and Drug Administration (FDA). Reference laboratories using laboratory-developed nucleic acid amplification tests have reported

sensitivities of 80% to 100% for *L. pneumophila* in lower respiratory tract specimens and specificities of greater than 90%. Diagnosis of Pontiac fever is usually based on laboratory findings, environmental studies, and epidemiology. Some outbreaks seemed linked to *L. pneumophila* serogroup 1. The *Legionella* urinary antigen test is commonly used for the diagnosis of Legionnaires' disease, but based on various studies, this test has not been useful for Pontiac fever.

Specimen collection and handling

Specimens for culture and direct examination commonly include sputum, bronchoalveolar lavage, and bronchial washings. Sputum scoring systems of expectorated sputum are not advised because of limited purulence. Other tissues or fluids, such as pleural fluid, are generally acceptable when suspicion is high. Small pieces of tissue may be overlaid with sterile water for transport to the laboratory; however, saline or buffer should not be used because of the inhibitory effects of sodium on *Legionella* spp. When delays of more than 2 hours between collection and processing are unavoidable, specimens should be refrigerated to avoid overgrowth of contaminating microbiota. It is recommended to freeze specimens at -70°C if processing will be delayed for several days.

Automated blood cultures are often insensitive, although lysis centrifugation using the Isolator system (Wampole Laboratories, Cranbury, NJ) is a good alternative, albeit labor-intensive. Urine is an important specimen to be collected for antigen detection, but it only detects *L. pneumophila* serogroup 1. Specimens are collected in sterile, leakproof containers. Specimens can be held at room temperature for a few hours; however, if processing is delayed, they should be stored at 2° to 8°C or frozen at -20°C . Environmental sources may be cultured during epidemiologic investigations, but this is normally a public health rather than a clinical or diagnostic function.

Microscopic examination

Legionella spp. are pleomorphic, weakly staining, gram-negative bacilli that are approximately 1 to $2\mu\text{m} \times 0.5\mu\text{m}$ in size. Extending the safranin counterstaining time to at least

10 minutes can enhance the staining intensity of the organisms. *L. micdadei* is weakly acid fast in tissue and stains best with the modified Kinyoun procedure (see Chapter 7). Other stains, including Diff-Quik (Baxter Scientific, Kansas City, MO) and Giemsa, can be used to facilitate observation of the organisms.

Nonspecific staining methods are most useful for examination of specimens from normally sterile sites. The faint-staining, pleomorphic gram-negative bacilli can be found outside of and within macrophages and segmented neutrophils (Fig. 18.25). The modified Kinyoun procedure can be used for tissue if *L. micdadei* is suspected.

Isolation and identification

Isolation methods

The most important test for Legionnaires' disease is culture of the organism. This should always be attempted, even when employing a rapid test such as urine antigen detection. Acid treatment of specimens contaminated with other bacteria, such as sputum, before inoculation enhances isolation of *Legionella* spp. (Fig. 18.26). In this procedure, an aliquot of the specimen is first diluted 1:10 with 0.2N potassium chloride-hydrochloric acid (KCl-HCl) (pH 2.2) and allowed to stand for no more than 4 minutes. The medium is then inoculated with a portion of the acid-treated specimen. Even specimens from normally sterile sites should be diluted 1:10 in tryptic soy broth or distilled water to dilute microbial inhibitors, such as complement, antibodies, and antimicrobial agents.

Legionella spp. are fastidious, aerobic bacteria that do not grow on SBA and require L-cysteine for growth. Tiny colonies may appear on CHOC agar that contains L-cysteine; however, BCYE with 0.1% α -ketoglutaric acid (BCYE α) agar is best for *Legionella* isolation. BCYE α agar is available commercially as nonselective and selective media, and both should be used for optimal isolation. Selective BCYE α media contain polymyxin B, anisomycin (an antifungal agent), and either vancomycin or cefamandole (e.g., BMPA, which contains cefamandole, polymyxin B, and anisomycin). Although selective medium improves recovery of *Legionella* spp. from highly contaminated specimens, it can inhibit growth of some *Legionella* spp.; therefore it should not be used alone. Inoculated media are incubated at 35°C in air; increased CO_2 can enhance the growth of some of the more fastidious species. *Legionella* spp. colonies are generally visible within 3 to 5 days, although some rarely isolated species might require 14 days.

Colony morphology

On BCYE α agar, colonies appear as grayish white or blue-green, convex, and glistening, measuring approximately 2 to 4 mm in diameter. When colonies are viewed with a dissecting microscope illuminated from above, they reveal a characteristic appearance (Fig. 18.27). The central portion of young colonies has a "ground-glass" appearance, light gray and granular, whereas the periphery of the colony has pink or light blue or bottle green bands with a furrowed appearance. Plates should be examined daily because older colonies lose these characteristic features and may be mistaken for other bacteria. Although some species autofluoresce with a long-wave ultraviolet light (366 nm), the variety of

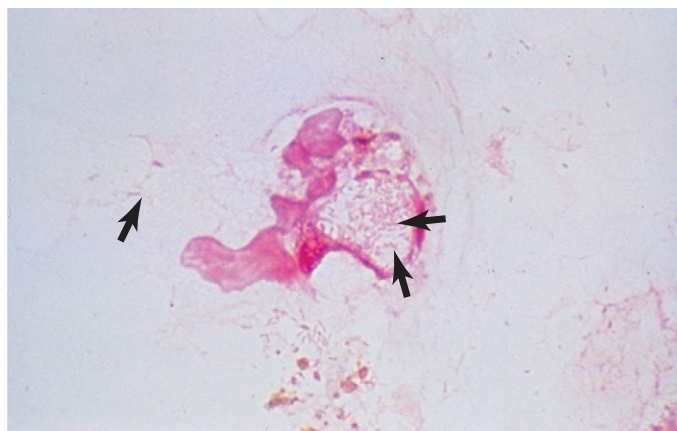


Fig. 18.25 Gram-stained smear of specimen demonstrating intracellular and extracellular *Legionella pneumophila* (arrows) ($\times 1000$).

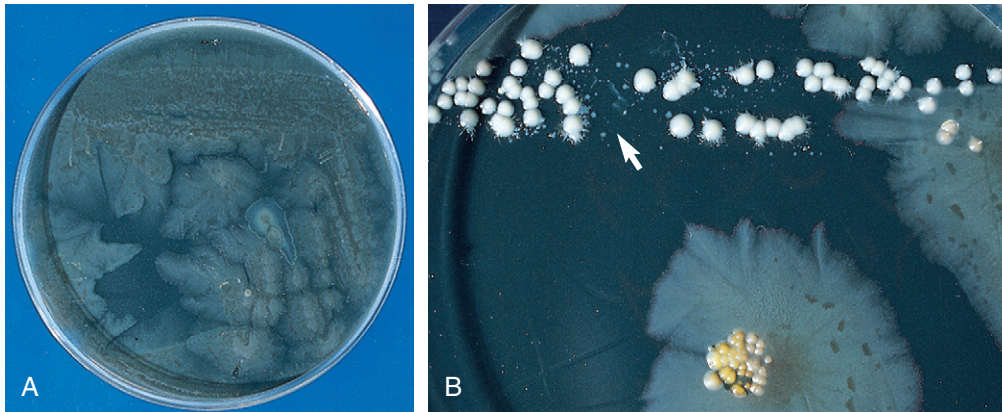


Fig. 18.26 **A**, Nonselective buffered charcoal yeast extract with 0.1% α -ketoglutaric acid (BCYE α) agar plate inoculated with a sputum specimen. Note overgrowth of respiratory biota. **B**, Selective BCYE α agar plate inoculated with the same sputum specimen, which has been acid washed before inoculation. Much of the respiratory biota has been eliminated. *Legionella* colonies are the smallest ones (arrow) in the first quadrant. (Courtesy Richard Brust.)

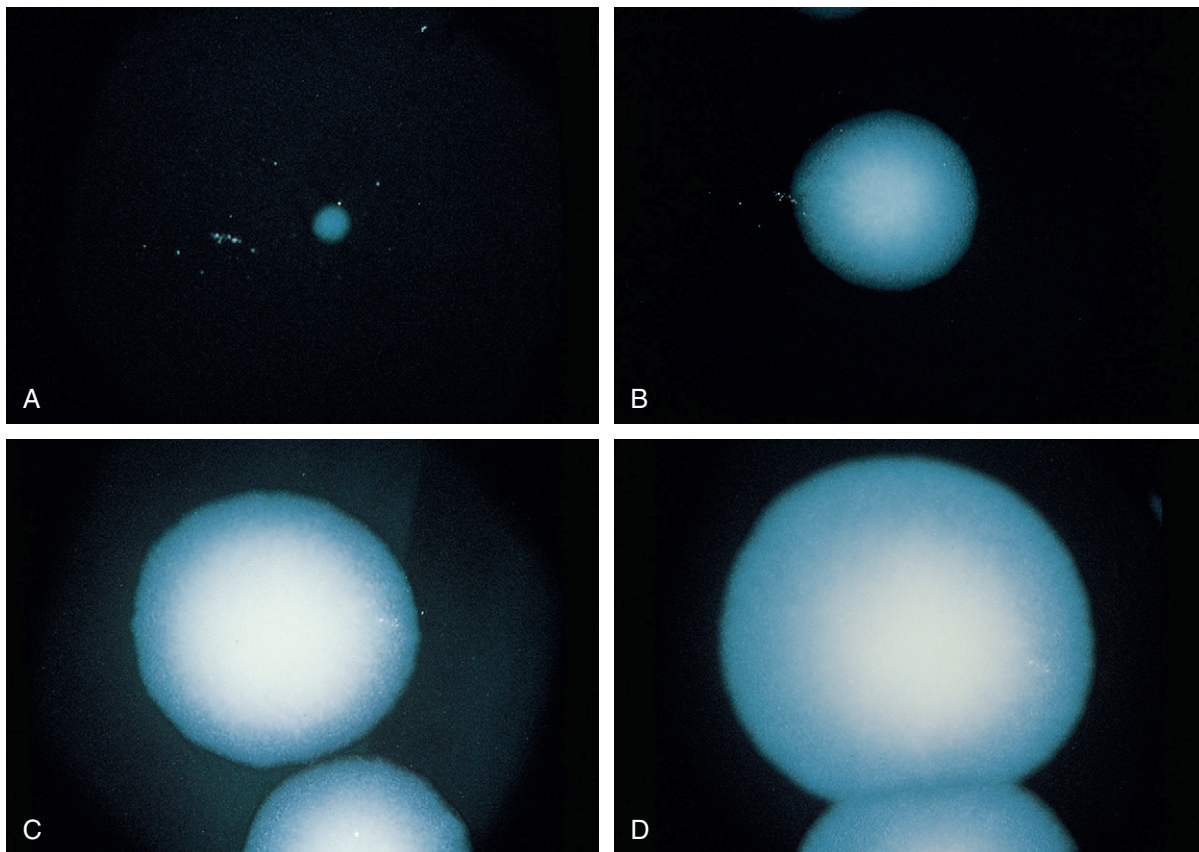


Fig. 18.27 **A**, A *Legionella pneumophila* colony on buffered charcoal yeast extract agar with 0.1% α -ketoglutaric acid after 3 days of incubation, viewed with a dissecting microscope ($\times 20$). **B**, The same colony after 4 days of incubation. **C**, The same colony after 5 days of incubation. **D**, The same colony after 7 days of incubation.

BOX 18.1 Common phenotypic characteristics of *Legionella* species

- Slow growth (3–5 days)
- Characteristic “ground-glass” colony morphology
- Lightly staining, gram-negative bacillus
- Requires L-cysteine for primary isolation
- No growth on unsupplemented sheep blood agar
- Asaccharolytic
- Catalase or oxidase: weakly positive

colors and number of species may limit the utility in a typical clinical laboratory.

Identification methods

Conventional methods

The physical and biochemical properties of *Legionella* spp. are listed in Box 18.1. The identification of suspected colonies as belonging to the genus *Legionella* can be accomplished by demonstrating L-cysteine requirement and/or performing

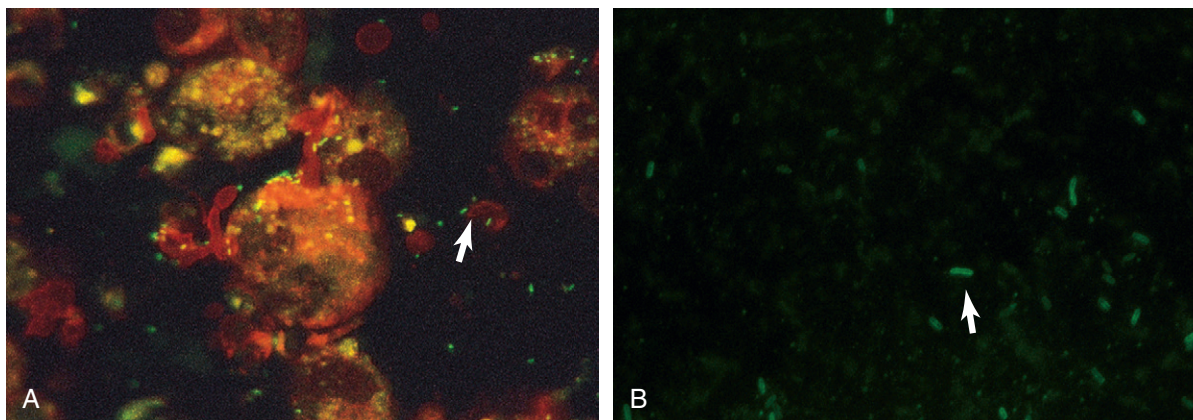


Fig. 18.28 **A**, *Legionella pneumophila* (arrow) in a specimen smear stained using the direct fluorescent antibody (DFA) technique ($\times 450$). **B**, *Legionella pneumophila* (arrow) in a specimen smear stained using the immunofluorescence technique ($\times 1000$). Note the intense peripheral staining of the organisms.

MALDI-TOF mass spectrometry. An FDA-approved immunofluorescent antibody test (Fig. 18.28) for all *L. pneumophila* serogroups is also available. Further biochemical testing has limited value, and identification of isolates to the species level is usually not necessary. MALDI-TOF mass spectrometry is dependent on libraries in the FDA-approved and/or research use only databases, and this method is frequently successful. Definitive identification of less common species is usually performed at reference or public health laboratories, frequently using 16S ribosomal ribonucleic acid (rRNA) or next-generation sequencing methodology.

Rapid methods

Urine antigen test

The FDA has approved several microplate enzyme immunoassay and rapid immunochromatographic assays for *Legionella* antigen detection in urine specimens. The rapid immunochromatographic assays detect soluble antigen from *L. pneumophila* serogroup 1. Tests generally have reported sensitivities and specificities of 95% or higher. Antigens can be detected by day 3 of the infection and can persist for 1 year; consequently, the test is of limited value in persons with a recent history of *Legionella* infection.

Enzyme immunoassays are used by many clinical laboratories. Compared with culture, when using concentrated urine, the sensitivity of enzyme immunoassays ranges from 90% to 94%, and the specificity ranges from 97% to 100%. Because *Legionella* species other than *L. pneumophila* serogroup 1 cause infections, it is important to confirm results with culture. In addition to persistent antigenuria following clinical disease, as mentioned earlier, prolonged secretion has been associated with immunosuppression, renal failure, and chronic alcoholism. Conversely, early antimicrobial intervention with macrolides may decrease antigen excretion in some patients. Despite some limitations, these rapid tests represent an important step forward in timely diagnosis of *Legionella* infections.

Serologic testing

The indirect fluorescent antibody (IFA) assay is the most common method employed for serologic diagnosis of legionellosis, although other methods, such as enzyme-linked immunosorbent assay, are available. For IFA, heat-killed or formalin-killed

bacteria are fixed to a microscope slide. Higher antibody titers can be seen with heat-treated organisms; however, less cross-reactivity occurs with formalin preparations. Cross-reacting immunoglobulins have been reported in patients with an infection caused by gram-negative bacilli, *Mycoplasma*, or *Chlamydia*. Only about 50% of patients with Legionnaires' disease seroconvert within 2 weeks. The sensitivity of serologic tests is reported to be 75% to 80%, with a specificity of 90% to 100%.

Because some patients develop IgM-only, IgG-only, or IgA-only responses, and IgM levels can persist for months, it is best to measure total antibody in acute and convalescent serum. A fourfold rise in titer to a titer of at least 1 : 128 is the most accurate method to diagnose infection by serologic testing. Serology is most useful for epidemiologic purposes because of its retrospective nature.

Antimicrobial susceptibility

Susceptibility testing of *Legionella* spp. is not standardized or routinely performed. When infections are diagnosed early, they can usually be treated successfully with a macrolide, ketolide, or doxycycline. Early diagnosis and treatment are particularly important in healthcare-associated infections because they usually involve immunocompromised patients, and the disease often takes an aggressive course.

Bordetella

Both *Bordetella pertussis* and *Bordetella parapertussis* are primary human pathogens of the respiratory tract, causing **whooping cough** or **pertussis**, although the latter organism is usually associated with a milder form of the disease. At least 12 other species are recognized, and about 11 in total have been found in humans. *Bordetella bronchiseptica* and *Bordetella avium* are respiratory tract pathogens of wild and domestic birds and mammals and are generally nonfastidious and recoverable on routine microbiological culture media. *B. bronchiseptica* is an opportunistic human pathogen, causing respiratory and wound infections. *Bordetella holmesii* and *Bordetella trematum* are more recently described species and are agents of immunocompromised bacteremia (*B. holmesii*) and wound or ear infection (*B. trematum*). *Bordetella ansorpii* has been reported as a rare cause of septicemia.

General characteristics

Members of the genus *Bordetella* are small, gram-negative bacilli or coccobacilli. All are obligate aerobic bacteria, grow best at 35° to 37° C, do not ferment carbohydrates, oxidize amino acids, are relatively inactive in biochemical test systems, and produce catalase, although this is variable in *B. pertussis*. *B. pertussis* is fastidious and requires special collection and transport systems and culture media. *B. pertussis* is inhibited by fatty acids, metal ions, sulfides, and peroxides, constituents found in many media. Media for the isolation of *B. pertussis* require protective substances, such as charcoal, blood, or starch, to bind and neutralize inhibitory substances. The other *Bordetella* spp., except for *B. parapertussis*, are less fastidious and grow on MAC agar or media containing blood.

Clinically significant species

Bordetella pertussis and *Bordetella parapertussis*

Virulence factors

B. pertussis possesses various virulence factors that play a role in the pathogenesis of disease. **Filamentous hemagglutinin** (FHA) and pertactin (a 69-kilodalton [kDa] outer membrane protein) are believed to facilitate attachment to ciliated epithelial cells. **Pertussis toxin** (PT) is a protein exotoxin that produces a wide variety of responses in vivo. The main activity of PT is modification of host proteins by adenosine diphosphate-ribosyl transferase, which interferes with signal transduction. *B. parapertussis* and *B. bronchiseptica* contain the structural gene for PT but do not express the complete operon. *B. pertussis* also produces adenylate cyclase toxin, which inhibits host epithelial and immune effector cells by inducing supraphysiologic concentrations of cyclic adenosine monophosphate. **Tracheal cytotoxin** contributes to pathogenesis by causing ciliostasis, inhibiting DNA synthesis, and promoting cell death. Other virulence factors have been proposed, but their roles in disease are unclear.

Clinical manifestations

Classic pertussis resulting from *B. pertussis* infection occurs after exposure to the organism through the respiratory tract. There is a 1- to 3-week incubation period, usually 7 to 10 days. The symptoms of the initial **catarrhal phase** are insidious and nonspecific and include sneezing, mild cough, runny nose, and perhaps conjunctivitis. At this stage, the infection is highly communicable because of the large number of organisms in the upper respiratory tract. However, cultures are not often performed at this stage because the symptoms are nonspecific.

The **paroxysmal phase** follows the catarrhal phase. The hallmark of this phase is the sudden onset of severe, repetitive coughing followed by the characteristic “whoop” at the end of the coughing spell. The whooping sound is caused by the rapid gasp for air following the prolonged bout of coughing. Coughing spells may occur many times a day and are sometimes followed by vomiting. Young children might experience apnea, pneumonia, or both and require aid in maintaining a patent airway. Many of these symptoms may be either absent or altered in very young infants, partially immunized children, or adolescents and adults. *B. parapertussis* generally causes a similar disease with milder symptoms. The **convalescent**

phase of disease generally begins within 4 weeks of onset with a decrease in frequency and severity of the coughing spells. Complete recovery may require weeks or months. Adults may or may not experience respiratory symptoms, which can range in severity from persistent cough to acute exacerbation of chronic bronchitis.

Epidemiology

Pertussis is a human disease; no animal reservoir or vector has been found. Infections caused by *Bordetella* spp. are acquired through the respiratory tract via respiratory aerosols or direct contact with infectious secretions. Organisms are uniquely adapted to adhere to and replicate on ciliated respiratory epithelial cells. The organisms remain localized to the respiratory tract, but toxins and other virulence factors that can have systemic effects are produced.

Pertussis is one of the most highly communicable diseases of childhood; secondary attack rates of 80% occur among susceptible contacts. Of all childhood diseases for which universal vaccination is recommended, only pertussis has an increased rate of incidence over the past decade. There were 18,617 cases of pertussis reported to the CDC in 2019. It is believed that many pertussis infections are not diagnosed; therefore cases are underreported. Infants younger than 1 year of age, who are too young to be fully vaccinated, continue to have the highest incidence of disease and are at greatest risk for severe disease. Adolescents and adults serve as reservoirs when their immunity from acellular pertussis (aP) vaccine, given in combination with the diphtheria and tetanus toxoids (DTaP), is either immature or waning.

The childhood vaccination series consists of five doses from 6 weeks to 6 years of age, and decreases in immunization rates lead to increased attack rates or major epidemics. Immunity is short lived, and *B. pertussis* appears to be maintained in the human population by adults who become transiently colonized. Single-dose booster vaccines, administered in combination with diphtheria and tetanus toxoids, are available for use in both adolescents and adults. If a child has recovered from confirmed pertussis, additional doses of pertussis vaccine are unnecessary.

Miscellaneous species

The remaining *Bordetella* spp. are opportunistic human pathogens. *B. bronchiseptica* is a respiratory tract pathogen of numerous animals, including dogs, in which it causes kennel cough. Symptomatic *B. bronchiseptica* infections in humans generally manifest with a nonspecific cough or bronchitis, and often patients have underlying conditions, such as immunosuppression or contact with animals. *B. bronchiseptica* and *B. holmesii* have been infrequently associated with pertussis syndrome and other respiratory tract infections.

Laboratory diagnosis

Contemporary laboratory diagnosis of pertussis generally employs culture isolation with or without PCR testing. DFA assays have low sensitivity and are not recommended. Other stains also have no role in diagnosis. Organisms do not survive well outside the host, so culture can also lack sensitivity.

Specimen collection and handling

Nasopharyngeal aspirates or swabs of Dacron polyester with a nonwire shaft constitute the specimen of choice for culture and PCR testing for *Bordetella*. Flocked swabs (FLOQSwab; Copan Diagnostics, Murrieta, CA) tend to have higher sensitivities when attempting to recover respiratory pathogens. Throat cultures have a much lower sensitivity and should be discouraged. Generally, two swabs are collected, one through each of the external nares; the swabs should be inserted as far back as possible into the nasopharynx, rotated, held a few seconds, and then gently withdrawn. In practice, swabs are used more commonly than aspiration methods.

Nasopharyngeal swab specimens should be plated directly onto culture media or transferred to an appropriate transport system at the bedside. Transport media, such as casamino acid (1% casein hydrolysate) or Amies with charcoal, can preserve viability up to 48 hours. Swabs for PCR can be transported dry at room temperature. Specimens for culture should be transported at room temperature and transferred to culture media as soon as they arrive at the laboratory.

Nucleic acid detection

Nucleic acid amplification methods can circumvent many of the problems associated with specimen transport and bacterial cultivation. Detection of *B. pertussis* DNA by PCR amplification has gained considerable use, but a lack of standardization and single target testing has also led to false-positive results and outbreaks mistakenly attributed to pertussis. Laboratories should use at least two DNA targets (e.g., *IS481* and *ptxP*) and may need to seek confirmation to avoid the mischaracterization of respiratory illness. Some PCR targets are not only found in *B. pertussis* but also in *B. parapertussis*, *B. holmesii*, and *B. bronchiseptica*. However, PCR assays are generally regarded as more sensitive than cultures and DFA assays. Some PCR kits including multiplex assays have received FDA approval.

Isolation and identification

Isolation methods

Since the original development of **Bordet-Gengou potato infusion agar** with glycerol and horse or sheep blood, few other formulations have been as successful. Alternative media include Regan-Lowe medium, which contains charcoal, starch, horse blood, cephalixin, and amphotericin B, and Stainer-Scholte medium, which contains casamino acids. Regan-Lowe medium is identical in composition to the transport medium of Regan and Lowe, except that it contains agar at full strength. The medium has a shelf life of 8 weeks and is commercially available. Care should be taken to ensure the appropriate concentration of cephalixin in the medium. Some strains of *B. pertussis* have been reported to be inhibited at 40 mg/L or greater. For this reason, it may be advisable to also plate a medium without cephalixin.

Plates for the recovery of *Bordetella* spp. should be incubated at 35°C in ambient air for a minimum of 7 days. It is important to ensure adequate moisture during this period to prevent plates from drying out. Small colonies of *B. pertussis*

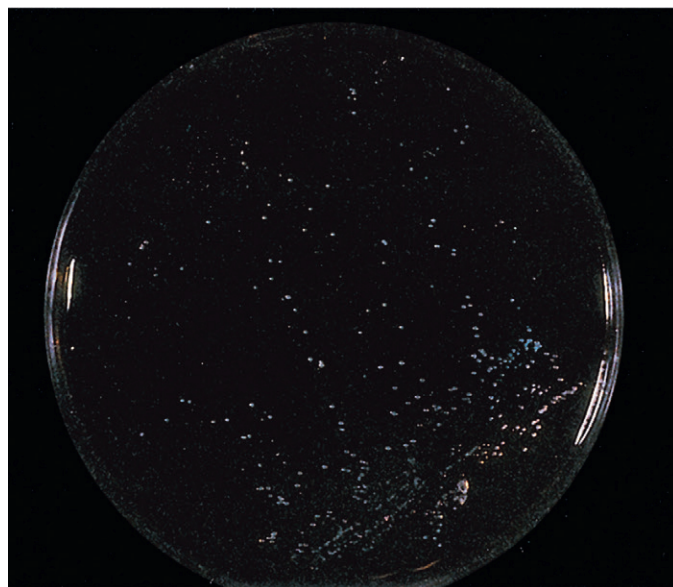


Fig. 18.29 Five-day-old *Bordetella pertussis* colonies on charcoal-horse blood agar (incident light from lower-right corner).

are detected in 3 to 7 days, whereas *B. parapertussis* is detected between days 2 and 3. A stereomicroscope should be used to detect the colonies before they become visible to the unaided eye.

Colony morphology

On charcoal-horse blood and Regan-Lowe media, young colonies are smooth, glistening, and silver, resembling mercury droplets (Fig. 18.29). Colonies turn whitish gray as they age. On Bordet-Gengou agar, colonies of *B. pertussis* and *B. parapertussis* are hemolytic.

Identification methods

On Gram stain of culture isolates, the organisms appear as tiny gram-negative coccobacilli and may become elongated if recovered from media containing cephalixin. It may be necessary to increase the safranin counterstaining time to 2 minutes to see typical morphology. Because of the fastidious nature of these organisms, identification by biochemical testing is difficult. Growth patterns should be observed, and biochemical tests can be performed (Table 18.8) to confirm the identification. Box 18.2 summarizes the laboratory diagnosis of pertussis by culture. MALDI-TOF mass spectrometry and 16S rRNA gene sequencing provide accurate identification, depending on the databases used.

Serologic testing

Serologic diagnosis of pertussis can be used to study outbreaks and to document seroconversion after immunization or infection. In addition, serologic testing can identify more cases in older vaccinated children, adolescents, and adults. However, assays cannot distinguish an immune response between vaccination or infection. In addition, serology cannot identify *B. parapertussis* infection. Detecting antipertussis toxin antibody by enzyme immunoassay or bead-based assays is recommended for diagnosing exposure to *B. pertussis*. Demonstrating a fourfold rise in titer in paired sera is

Table 18.8 Differential characteristics of *Bordetella* species infecting humans

Characteristics	<i>B. pertussis</i>	<i>B. parapertussis</i>	<i>B. bronchiseptica</i>
Growth on			
Charcoal–horse blood	+ (3–5 days)	+ (2–3 days)	+ (1–2 days)
Blood agar	–	+	+
MacConkey agar	–	–	+
Catalase	+	+	+
Oxidase	+	–	+
Urease production	–	+ (24 hours)	+ (4 hours)
Nitrate reduction	–	–	+
Motility	–	–	+

BOX 18.2 Summary of laboratory diagnosis of pertussis by culture

- Using Dacron swabs or flocked swabs, collect two nasopharyngeal specimens.
- Transfer to transport system:
 - Casamino acid (1% casein hydrolysate)
 - Amies transport medium with charcoal
 - Regan-Lowe transport medium
- Perform polymerase chain reaction (PCR) test for *Bordetella pertussis*.
- Inoculate Bordet-Gengou, Regan-Lowe, or Stainer-Scholte medium. Incubate medium in a moist chamber (without carbon dioxide [CO₂]) at 35° C for 7 days.
- Examine plates using a stereomicroscope for typical colonies.
- Screen *B. pertussis*/*B. parapertussis*-like colonies with Gram stain, and use matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry or 16S rRNA gene sequencing to confirm.

optimal for diagnosis. A single IgG antibody titer greater than 100 IU/mL is indicative of recent infection, whereas a titer less than 40 IU/mL is not. Assays are in need of better standardization. Detecting IgM is not recommended.

Antimicrobial susceptibility

B. pertussis and *B. parapertussis* are generally sensitive to the macrolides, ketolides, penicillins, and tetracyclines but are resistant to oral cephalosporins. Although a few strains of erythromycin-resistant *B. pertussis* have been reported, erythromycin, a macrolide, is still recommended for pertussis treatment and prophylaxis. Erythromycin is important for eradication of the organism and prevention of secondary cases, but it has clinical efficacy only if treatment is started during the catarrhal phase of disease. Azithromycin has fewer and milder side effects, has a longer half-life, and requires fewer daily doses, resulting in better patient compliance. Trimethoprim-sulfamethoxazole is also an alternative for treatment or prophylaxis. Routine antimicrobial susceptibility testing of *B. pertussis* or *B. parapertussis* is unnecessary because of predictable sensitivity to the macrolides. The susceptibility of *B. bronchiseptica* is unpredictable, and tests should be performed, although the organism is usually susceptible to the aminoglycosides.

POINTS TO REMEMBER

- The genus *Haemophilus* consists of gram-negative coccobacilli or bacilli. They are facultative anaerobes that are generally oxidase and catalase positive.
- *Haemophilus* spp. require preformed growth factors (X and V factors) present in blood. Testing for these factors with X and V strips and the porphyrin test are important aspects of laboratory identification procedures.
- The advent of the Hib vaccine has decreased infection by *H. influenzae* type b in children in the United States by 99%, but serotype b remains prevalent in developing countries.
- *H. ducreyi* causes the STD chancroid. In contrast with many other *Haemophilus* spp., it is not considered part of the normal oral biota of humans.
- HACEK is an acronym for *Haemophilus* spp., *Aggregatibacter* spp., *Cardiobacterium hominis*, *Eikenella corrodens*, and *Kingella* spp. The HACEK group is an important cause of endocarditis, specifically related to poor oral hygiene or dental procedures.
- *Pasteurella*, *Brucella*, and *Francisella* spp. are important causes of zoonotic infections.
- The most common *Pasteurella* species isolated from humans, *P. multocida*, most frequently causes wound infections after cat or dog bites.
- *Brucella* and *Francisella* spp. are fastidious gram-negative coccobacilli causing zoonoses and are considered potential bioterrorism agents.
- *Legionella* spp. are pleomorphic, weakly staining gram-negative bacilli. They are transmitted to humans primarily via aerosolized particles. Sources include contaminated potable water distribution systems, respiratory therapy equipment, and recreational waters. Transmission among humans has not been demonstrated.
- Legionnaires' disease is a febrile illness with pneumonia that is often associated with travel. Pontiac fever is a milder febrile disease resembling influenza that may be caused by inhalation of bacterial toxin.
- On BCYE α agar, *L. pneumophila* colonies appear convex and glistening. The central portion of young colonies have a "ground-glass" appearance (light gray and granular), whereas the periphery of the colony has pink, light blue, or bottle-green bands.
- Using a combination of nonselective and selective BCYE α agar is the best strategy for isolation of *Legionella* organisms. Acid treatment of specimens contaminated with other bacteria before inoculation enhances isolation of *Legionella* spp.
- A rapid assay useful for the detection of *Legionella pneumophila* serogroup 1 is the urine antigen test.
- *B. pertussis* and *B. parapertussis* cause pertussis, or whooping cough, and are primary human pathogens of the respiratory tract. They are acquired through the respiratory tract via respiratory aerosols or direct contact with infectious secretions.
- *Bordetella* spp. are tiny, gram-negative coccobacilli and might become elongated if recovered from media containing cephalixin.
- For the detection of *Bordetella* spp., nasopharyngeal aspirates or swab specimens should be plated directly onto culture media or transferred to an appropriate transport system (casamino acid or Amies transport medium) at the bedside.
- Contemporary laboratory diagnosis of pertussis generally employs culture isolation with or without PCR testing.

- *B. bronchiseptica* can be part of the normal oral biota of dogs and cats, and infections in humans typically follow bites by these animals.
- Antimicrobial susceptibility testing is not normally performed on *B. pertussis*. The drug of choice in the treatment of pertussis is erythromycin.

LEARNING ASSESSMENT QUESTIONS

- What are the growth patterns of *Haemophilus influenzae* with the X and V strip test?
- A microbiologist is reading a sputum culture and notices tiny, translucent colonies growing closely around a β -hemolytic colony on sheep blood agar (SBA). The colonies are growing in the area where the blood has been hemolyzed. What is the probable identity of the organism that is growing closely to the β -hemolytic organism?
 - Neisseria* spp.
 - Haemophilus* spp.
 - Acinetobacter* spp.
 - Moraxella* spp.
- Which bacterium is the causative agent of kennel cough in dogs and is occasionally associated with respiratory tract infections in humans?
 - Bordetella bronchiseptica*
 - Pasteurella multocida*
 - Francisella tularensis*
 - Capnocytophaga canimorsus*
- What are the optimal growth conditions for the recovery of *Haemophilus ducreyi*?
- How would you compare the pathogenesis of *Haemophilus aegyptius* with *Haemophilus influenzae* biogroup *aegyptius*?
- A cervical culture for possible gonococcal infection is sent to the microbiology laboratory. After 24 hours of incubation, the modified Thayer-Martin plate has small opaque colonies that adhere slightly to the medium. Microscopic examination reveals gram-negative coccobacilli, many with square ends. The organism ferments glucose and is superoxol and catalase negative. What is the most likely identification of the organism?
 - Neisseria gonorrhoeae*
 - Kingella denitrificans*
 - Moraxella catarrhalis*
 - Haemophilus ducreyi*
- A 52-year-old male patient who had recently received a kidney transplant was admitted to the hospital with a low-grade fever, a heart murmur, and neutropenia. He had a history of periodontal disease and recently had had two teeth extracted. Blood cultures were positive after 48 hours. The isolate grew on chocolate agar and SBA in 5% CO₂. The colonies were nonhemolytic, slightly adhered to the surface of the media, and had a slight yellow appearance when removed. The isolate was catalase, indole, and oxidase negative. Microscopic morphology indicated gram-negative fusiform bacilli. What is the most probable identification of this organism?
 - Aggregatibacter aphrophilus*
 - Kingella kingae*
 - Cardiobacterium hominis*
 - Capnocytophaga* spp.
- An isolate from an infected cat bite is oxidase, catalase, ornithine decarboxylase, indole, and urease negative. After 48 hours of incubation at 35° C, growth on SBA was described as mucoid colonies exhibiting a greenish-brown halo. A McConkey agar plate showed no growth. Which organism is the mostly likely cause of the infection?
 - Pasteurella multocida*
 - Bordetella bronchiseptica*
 - Francisella tularensis*
 - Capnocytophaga canimorsus*
- Which microbiologic tests are most useful in differentiating *Brucella melitensis* from *Haemophilus influenzae*?
- A patient is complaining of a painful cervical lymph node following a case of pharyngitis. Further investigation reveals that the patient consumed a medium-cooked wild rabbit in a restaurant in Germany 2 months earlier. What is the most likely cause of the infection?
- Which risk factors contribute to the more severe form of legionellosis?
- Which environmental factors contribute to infection caused by *Legionella* spp.?
- What is the culture medium of choice for the recovery of *Legionella* spp.?
- Which factors of *Legionella* can contribute to the colonization of human-made water supplies?
- What is the best nonrespiratory specimen for rapid detection of *Legionella*?
- Which presumptive identification methods are currently used to identify *Legionella* spp. in culture?
- Besides respiratory tract specimens, which clinical specimen is useful for the sensitive detection of *Legionella* antigen?
 - Blood
 - Stool
 - Urine
 - Cerebrospinal fluid (CSF)
- Are adults immune to *Bordetella pertussis* infection? Explain your answer.
- What are the clinical samples of choice for the diagnosis of *Bordetella pertussis* infection?
- Which transport media are appropriate for maximum recovery of *Bordetella pertussis*?
- Which method is preferred for the rapid detection of *Bordetella* in nasopharyngeal specimens?
- How would you compare the diseases caused by *Bordetella pertussis* and *B. parapertussis*?
- Is serology a good method to identify and respond to pertussis outbreaks in real time?
- What is the antimicrobial agent of choice for the treatment of pertussis?
 - Ampicillin
 - Amoxicillin
 - Erythromycin
 - Penicillin
- Bordetella bronchiseptica* is considered normal oral biota in which of the following?
 - Humans
 - Dogs
 - Cows
 - Rats

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Enterobacterales

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CHAPTER OUTLINE

General characteristics, 418

- Microscopic and colony morphology, 418
- Classification, 418
- Virulence and antigenic factors, 419
- Clinical significance, 419
- Antimicrobial resistance in the Enterobacterales, 419

Opportunistic members of the order Enterobacterales and associated infections, 421

- Escherichia coli*, 421
- Klebsiella*, 426
- Enterobacter* and *Cronobacter*, 427
- Citrobacter*, 428
- Kluyvera*, 429
- Plesiomonas*, 429
- Erwinaceae, 430
- Morganellaceae, 430
- Hafniaceae, 431
- Serratia*, 432

Primary pathogens in the order Enterobacterales, 432

- Salmonella*, 432
- Shigella*, 435

Other genera of the order Enterobacterales, 439

- Budvicia*, 439
- Buttiauxella*, 439
- Cedecea*, 439
- Ewingella*, 439
- Leclercia*, 439
- Leminorella*, 439
- Moellerella*, 439
- Rahnella*, 439
- Tatumella*, 439

Laboratory diagnosis of the Enterobacterales, 439

- Specimen collection and transport, 439

Isolation and identification, 440

Screening stool cultures for pathogens, 440

Serologic grouping, 442

Bibliography, 453

OBJECTIVES

After reading and studying this chapter, you should be able to:

1. Describe the general characteristics of organisms that belong to the order Enterobacterales.
2. Describe the antigenic structures of the order Enterobacterales.
3. Explain how antigenic structures are used for identification of clinically significant Enterobacterales.
4. Compare the virulence factors of the *Escherichia coli* strains pathogenic for the gastrointestinal tract and the *E. coli* strains involved in extraintestinal human diseases.
5. Compare the pathogenesis of the three species of *Yersinia* most often recovered from humans.
6. Describe the pathogenesis of the clinically relevant members of the order Enterobacterales.
7. Given an organism's characteristic growth on nonselective and selective differential media, presumptively identify the isolate to the genus level.
8. Match the species of *Shigella* to their appropriate serogroup.
9. Given the key reactions for identification, place an unknown organism in its proper family, genus, and species.
10. Develop an algorithm for the identification of the clinically significant Enterobacterales.

KEY TERMS

Bacillary dysentery

Buboes

Diffusely adherent *Escherichia coli* (DAEC)

Enteric

Enteraggregative *Escherichia coli* (EAEC)

Enterohemorrhagic *Escherichia coli* (EHEC)

Enteroinvasive *Escherichia coli* (EIEC)

Enteropathogenic <i>Escherichia coli</i> (EPEC)	Shiga toxin (Stx)
Enterotoxigenic <i>Escherichia coli</i> (ETEC)	Shiga toxin–producing <i>Escherichia coli</i> (STEC)
H antigen	Typhoid fever
K antigen	Traveler's diarrhea
O antigen	Verotoxin
	Vi antigen

Case in point

A 71-year-old male patient who was hospitalized for diabetic ketoacidosis complained of flank pain and painful urination. A urine sample was plated, and after 18 hours of incubation, a MacConkey (MAC) agar plate showed moderate growth of lactose-fermenting organisms. A sheep blood agar (SBA) plate revealed oxidase-negative, nonhemolytic colonies. The following biochemical results were obtained on the isolate: H₂S negative, indole, methyl red, Voges-Proskauer, citrate (IMViC) reactions were + + – +; urea was hydrolyzed; and arginine and ornithine were decarboxylated. Antimicrobial susceptibility test revealed resistance to cefotaxime, norfloxacin, ciprofloxacin, and the aminoglycosides.

Issues to consider

After reading the patient's case history, consider:

- The significance of this patient's health status and medical history
- The colony morphology feature that provides clues about the identity of the organism
- The biochemical tests that are the most specific for identification of this organism

Comprehensive genomic analysis with phylogenetic reconstructions of members of the heterogeneous family Enterobacteriaceae resulted in the creation of a new order, Enterobacterales. All members of the original family Enterobacteriaceae were placed in the new order. The order Enterobacterales was divided into nine families: Bruguierivoraceae, Budviciaceae, Enterobacteriaceae, Erwiniaceae, Hafniaceae, Morganellaceae, Pectobacteriaceae, Thorselliaceae, and Yersiniaceae. Genera remaining within the family Enterobacteriaceae include *Citrobacter*, *Cronobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Kluyvera*, *Raoultella*, *Salmonella*, *Shigella*, and several others. Despite the enormity and diversity of this order, clinical isolates in acute-care facilities consist primarily of only a few species. However, it is important to be aware of other species that cause significant human infectious diseases.

This chapter is divided into three major areas: (1) clinically significant species that cause opportunistic infections, (2) primary intestinal pathogens and their related human infections, and (3) methods of identification of these organisms. Among the many organisms in the order Enterobacterales, this chapter focuses only on the members that have been associated with human diseases.

General characteristics

The order Enterobacterales consists of numerous diverse organisms. They have several key laboratory features in common, but as 16S rRNA and deoxyribonucleic acid (DNA) studies on

these bacteria progress, classification of the members changes. Some organisms are added (e.g., *Enterobacter bugandensis*), new species are being discovered, and some may be removed from the order. Currently, several key characteristics help place an organism in the order Enterobacterales (Box 19.1).

Microscopic and colony morphology

Members of the order Enterobacterales are gram-negative, non-spore-forming, facultatively anaerobic bacilli. On Gram-stained smears, they can appear as coccobacilli or as straight rods. Colony morphology on nonselective media, such as SBA or chocolate (CHOC) agar, is of little value in initial identification. Except for certain members that produce characteristically large and very mucoid colonies (e.g., *Klebsiella* and sometimes *Enterobacter*), members of this order produce large, moist, gray colonies on nonselective media and are indistinguishable. However, many isolates of *E. coli* are β -hemolytic.

A wide variety of selective and differential media, such as eosin-methylene blue (EMB) and MAC agars, and highly selective media, such as Hektoen enteric (HE) and xylose-lysine-desoxycholate (XLD) agars, are available for presumptive identification of clinical pathogens. These media contain one or more carbohydrates, such as lactose and sucrose, which show the ability of the species to ferment specific carbohydrates. Acid production via fermentation is indicated by a color change on the medium, which results from a decrease in pH detected by a pH indicator present in the medium. Nonfermenting species are differentiated by lack of color change, with colonies retaining the original color of the medium. Species that produce hydrogen sulfide (H₂S) can be readily distinguished when placed on HE or XLD agar. HE and XLD agars contain sodium thiosulfate and ferric ammonium citrate, which produce blackening of H₂S-producing colonies. These features have been used to initially differentiate and characterize certain genera. Definitive identification depends on biochemical reactions, antigen determination, and, more recently, protein profiles via matrix-assisted laser desorption/ionization–time-of-flight (MALDI-TOF) mass spectrometry.

Classification

The order Enterobacterales belongs to the class Gammaproteobacteria. It includes over 60 genera and 250 species. Most of the species are placed into the family Enterobacteriaceae.

BOX 19.1 Key characteristics of the order Enterobacterales

- They are gram-negative, facultatively anaerobic, non-spore-forming bacilli and coccobacilli.
- They do not produce cytochrome oxidase, except *Plesiomonas*.
- They ferment glucose.
- They reduce nitrate to nitrite, except *Photobacterium* and *Xenorhabdus*.
- They are motile at 37°C, except *Klebsiella*, *Shigella*, and *Yersinia*.
- Except for *Klebsiella*, *Proteus*, and some *Enterobacter* isolates, none has remarkable colony morphology on laboratory media. They appear large, moist, and gray on sheep blood agar (SBA), chocolate agar, and most nonselective media. Most are nonhemolytic on SBA, except *E. coli*.

Table 19.1 lists bacterial species in the order Enterobacterales and their respective families. Table 19.2 shows the biochemical features of select genera. Although taxonomists use nucleic acid sequences, biochemical features are an easy and effective way of classifying bacteria into genera and is employed throughout this chapter.

Virulence and antigenic factors

Many factors affect the virulence of the Enterobacterales, such as the ability to adhere, colonize, produce toxins, and evade host defenses. Many pathogenic species of this order possess antigens that can be used in the identification of different serologic groups. These antigens include:

- **O antigen**, or somatic antigen: a heat-stable antigen located on the cell wall
- **H antigen**, or flagellar antigen: a heat-labile antigen found on the surface of flagella, the structures responsible for motility
- **K antigen**, or capsular antigen: a heat-labile polysaccharide found only in certain encapsulated species. Examples are the K1 antigen of *E. coli* and the **Vi antigen** of *Salmonella enterica* subsp. *enterica* serotype Typhi.

Clinical significance

Members of the order Enterobacterales are ubiquitous in nature. Some species exist as free-living organisms in water, soil, or sewage, and some are animal or plant pathogens. The medically important Enterobacterales, with few exceptions, share a common niche; they reside in the gastrointestinal (GI) tract and are commonly referred to as **enteric** bacilli. Except for *Salmonella*, *Shigella*, and *Yersinia*, they can be resident microbiota if confined to their natural environment.

Based on the clinical infections produced, members of the order Enterobacterales can be divided into two broad categories: (1) opportunistic pathogens and (2) primary pathogens. The opportunistic pathogens are often a part of the usual intestinal microbiota of both humans and animals. However, outside their normal body sites, these organisms can produce serious extraintestinal, opportunistic infections. For example, *E. coli*, one of the best-studied members of the family Enterobacteriaceae, is a member of the normal bowel biota but can cause urinary tract infections (UTIs), septicemia, wound infections in healthy individuals, and meningitis in neonates.

Other organisms can be equally devastating in immunocompromised hosts or when introduced to wounds from contaminated soil or water. *S. enterica*, *Shigella* spp., and *Yersinia* spp. are considered primary or true pathogens; that is, they are not present as commensal biota in the GI tract of humans. These organisms produce infections resulting from ingestion of contaminated food or water or from other sources. Table 19.3 lists some diseases associated with members of the order Enterobacterales.

Antimicrobial resistance in the Enterobacterales

In 2019, the Centers for Disease Control and Prevention (CDC) listed 18 drug-resistant threats to the United States categorized based on level of concern: urgent, serious, and

Table 19.1 Classification of selected species within the order Enterobacterales

Family	Genus	Species
Enterobacteriaceae	<i>Escherichia</i>	<i>coli</i>
		<i>albertii</i>
		<i>fergusonii</i>
		<i>dysenteriae</i>
		<i>flexneri</i>
	<i>Shigella</i>	<i>boydii</i>
		<i>sonnei</i>
		<i>freundii</i> complex
	<i>Citrobacter</i>	<i>koseri</i>
		<i>amalonaticus</i>
		<i>sakazakii</i>
	<i>Cronobacter</i>	<i>malonaticus</i>
		<i>farmeri</i>
	<i>Enterobacter</i>	<i>cloacae</i> complex
		<i>Klebsiella pneumoniae</i> subsp. <i>pneumoniae</i>
	<i>Klebsiella</i>	<i>pneumoniae</i> subsp. <i>ozaenae</i>
		<i>pneumoniae</i> subsp. <i>rhinoscleromatis</i>
		<i>aerogenes</i>
		<i>variicola</i>
		<i>Raoultella ornitholytica</i>
		<i>Salmonella enterica bongori</i>
		<i>Cronobacter sakazakii malonaticus</i>
Hafniaceae	<i>Edwardsiella</i>	<i>tarda</i>
		<i>hoshinae</i>
Morganellaceae	<i>Hafnia</i>	<i>alvei</i>
		<i>paralvei</i>
	<i>Morganella</i>	<i>morganii</i>
		<i>Proteus mirabilis</i>
		<i>vulgaris</i>
	<i>Providencia</i>	<i>penneri</i>
		<i>myxofaciens</i>
		<i>alcalifaciens</i>
		<i>rettgeri</i>
		<i>stuartii</i>
Yersiniaceae	<i>Serratia</i>	<i>marcescens</i>
		<i>liquefaciens</i>
		<i>rubidaea</i>
		<i>fonticola</i>
		<i>odorifera</i>
		<i>plymuthica</i>
		<i>pestis</i>
	<i>Yersinia</i>	<i>enterocolitica</i>
		<i>pseudotuberculosis</i>
		<i>frederiksenii</i>
		<i>kristensenii</i>
		<i>intermedia</i>
		<i>agglomerans</i>
Erwiniaceae	<i>Pantoea</i>	<i>agglomerans</i>

Modified from Schoch, C., et al. *NCBI taxonomy: a comprehensive update on curation, resources, and tools*. Database (Oxford). 2020: Baaa062.

Table 19.2 Biochemical characteristics of select genera of Enterobacterales

Tests or substrate	<i>Escherichia</i> and <i>Shigella</i>	<i>Edwardsiella</i>	<i>Citrobacter</i>	<i>Salmonella</i> ^a	<i>Klebsiella</i> , <i>Enterobacter</i> , and <i>Serratia</i>	<i>Proteus</i> , <i>Morganella</i> , <i>Yersinia</i> and <i>Providencia</i> ^b
H ₂ S (TSI agar)	–	+	+ or –	+	–	+ or –
Urease	–	–	(+ ^w) or –	–	– or (+)	+ or –
Indole	+ or –	+	– or +	–	–	+ or –
Methyl red	+	+	+	+	–	+
Voges-Proskauer	–	–	–	–	+	–
Citrate (Simmons)	–	–	+	+	+	d
KCN	–	–	+ or –	–	+	+
Phenylalanine deaminase	–	–	–	–	–	+
Mucate	d	–		d	+ or –	–
Mannitol	+ or –	–	+	+	+	– or +

^a*Salmonella* serotypes Typhi and Paratyphi and some other rare serotypes fail to use citrate in Simmons medium. Cultures of serotype Paratyphi and some rare serotypes may fail to produce H₂S.

^bSome cultures of *Proteus mirabilis* may yield positive Voges-Proskauer tests.

+, ≥90% positive within 1 or 2 days; (+), positive reaction after ≥3 days (decarboxylase tests: 3 or 4 days); –, ≥90% no reaction in 30 days; + or –, most cultures positive, some strains negative; – or +, most strains negative, some cultures positive; d, different reactions; +, (+), –, +^w, weakly positive reaction; H₂S, hydrogen sulfide; KCN, potassium cyanide; TSI, triple sugar iron.

Modified from Ewing, W. H. (1986). *Edwards and Ewing's identification of enterobacteriaceae* (4th ed). East Norwalk, CT: Appleton & Lange.

Table 19.3 Bacterial species and infections they commonly produce

Bacterial species	Diseases
<i>Citrobacter</i> spp.	Urinary tract
<i>Cronobacter</i> spp.	Opportunistic and healthcare-associated infections, wound infections, bacteremia, meningitis
<i>Edwardsiella</i> spp.	Diarrhea, wound infections, septicemia, meningitis, enteric fever
<i>Enterobacter</i> spp.	Opportunistic and healthcare-associated infections, wound infections, septicemia, bacteriuria
<i>Escherichia coli</i>	Bacteriuria, septicemia, neonatal sepsis, meningitis, diarrheal syndrome
<i>Hafnia alvei</i>	Diarrhea, bacteremia, respiratory tract infections
<i>Klebsiella</i> spp.	Opportunistic and healthcare-associated infections, bacteriuria, pneumonia, septicemia
<i>Morganella</i> spp.	Opportunistic and healthcare-associated infections
<i>Pantoeae</i>	Opportunistic and healthcare-associated infections
<i>Proteus</i> spp.	Bacteriuria, wound infections, septicemia
<i>Providencia</i> spp.	Opportunistic and healthcare-associated infections, wound infections, septicemia, bacteriuria
<i>Raoultella ornithinolytica</i>	Opportunistic and healthcare-associated infections, wound infections, septicemia, bacteriuria
<i>Salmonella</i> spp.	Septicemia, enteric fever, diarrhea
<i>Shigella</i> spp.	Diarrhea, dysentery
<i>Serratia</i> spp.	Opportunistic and healthcare-associated infections, wound infections, septicemia, bacteriuria
<i>Yersinia pestis</i>	Plague
<i>Yersinia pseudotuberculosis</i>	Mesenteric adenitis, diarrhea
<i>Yersinia enterocolitica</i>	Mesenteric adenitis, diarrhea

Modified from Forsythe, S. J., et al. (2019). *Klebsiella* and selected Enterobacterales. In K. C. Carroll, et al. (Eds.), *Manual of clinical microbiology* (12th ed., p. 724). Washington, DC: ASM Press.

concerning. Carbapenem-resistant Enterobacteriaceae (CRE; referring to the original family Enterobacteriaceae) was listed as one of five urgent concerns. Infections with CREs, particularly in health care settings, are often life-threatening. CRE is defined as resistance to imipenem, meropenem, doripenem, or

ertapenem, or documentation that the isolate possesses a carbapenemase. The Xpert Carba-R Assay (Cepheid, Sunnyvale, CA) is a real-time multiplex polymerase chain reaction (PCR) assay that rapidly detects five genes coding for carbapenemases. The assay has a sensitivity of 100% and a specificity of

>99%. Screening of hospitalized patients is recommended by many public health organizations to help contain the spread of carbapenemase-producing bacteria.

With the discovery of the plasmid-transmissible colistin marker *mcr-1* in a CRE strain in 2016, antimicrobial resistance in Enterobacterales continues to grow more serious. For example, an increasing number of *E. coli*, *K. pneumoniae*, and *Klebsiella oxytoca* clinical strains produce plasmid-mediated extended-spectrum β -lactamases (ESBLs) including carbapenemases, cephalosporinases, or metallo- β -lactamases, which can inactivate extended-spectrum cephalosporins. The *K. pneumoniae* carbapenemase (KPC), found in *Klebsiella* spp., *Enterobacter* spp., and *Serratia marcescens*, now joins VIM, IMP, New Delhi metallo- β -lactamase (NDM-1), and others in the growing arsenal of resistance genes found in CREs. For definitions and more information on antimicrobial classes, see [Chapter 12](#).

Opportunistic members of the order Enterobacterales and associated infections

Escherichia coli

E. coli, the most significant species in the genus *Escherichia*, was first described by Escherich in 1885. *E. coli* was initially considered a harmless member of the colon resident biota. It

is now recognized as an important human pathogen associated with a wide range of clinical syndromes, including UTIs, diarrheal diseases, and central nervous system infections. It is so commonly isolated from colon biota that *E. coli* is used as a primary marker of fecal contamination in water quality testing.

Most strains of *E. coli* are motile and generally possess adhesive fimbriae and sex pili, and O, H, and K antigens. *E. coli* O groups have shown remarkable cross-reactivity with O antigens from other members of the Enterobacteriaceae, most notably *Shigella* spp. This is one of the reasons *Escherichia* and *Shigella* spp. are grouped together in the family Enterobacteriaceae. Serotyping for both O and H antigens is often useful in strain identification, particularly strains associated with serious enteric disease. The K antigen often masks the O antigen during bacterial agglutination testing with specific antiserum. This phenomenon can make it difficult to determine the serotype.

On certain selective and differential media, such as MAC or EMB agars, *E. coli* has a distinctive morphology. It usually appears as a lactose-positive (pink) colony with a surrounding area of precipitated bile salts on MAC agar ([Fig. 19.1](#)). On EMB agar, it has a green metallic sheen. *E. coli* typically has the following properties:

- Fermentation of glucose, lactose, trehalose, and xylose
- Production of indole from tryptophan
- Glucose fermentation by the mixed acid pathway: methyl red positive and Voges-Proskauer negative

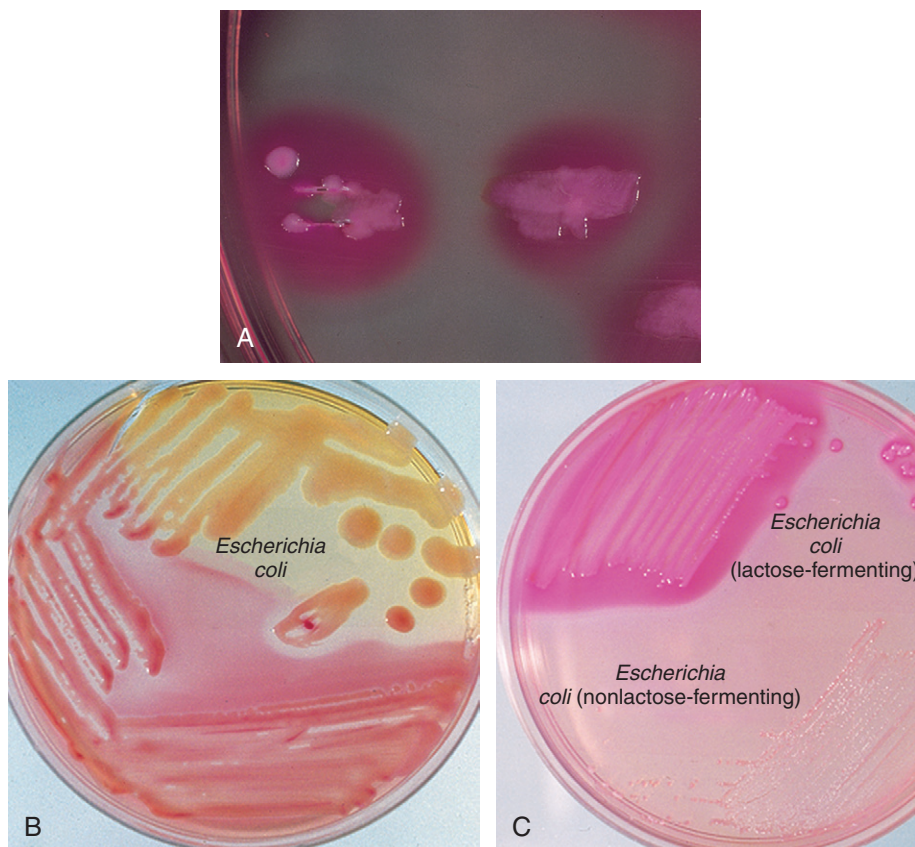


Fig. 19.1 A, Typical dry, lactose-positive *Escherichia coli* growing on MacConkey (MAC) agar. Note the pink precipitate surrounding the individual colonies. B, Mucoid colonies of *E. coli* growing on MAC agar. C, Nonlactose-fermenting *E. coli* compared with typical lactose-fermenting *E. coli* on MAC agar. (B and C, Courtesy Jean Barnishan.)

- Does not produce H_2S , deoxyribonuclease (DNase), urease, or phenylalanine deaminase
- Cannot use citrate as a sole carbon source

Extraintestinal *Escherichia coli* infections

E. coli is one of the most common causes of septicemia and meningitis among neonates, accounting for about 40% of the cases of early-onset meningitis. Similar infections resulting from this organism are uncommon in older children. A newborn usually acquires the infection in the birth canal just before or during delivery, when the mother's vagina is heavily colonized. Infection may also result if contamination of the amniotic fluid occurs.

The strains associated with diarrheal disease appear to be distinct from strains associated with neonatal sepsis or meningitis. The capsular antigen K1, present in some strains of *E. coli*, is the most documented virulence factor associated with neonatal meningitis and septicemia infections. It is present on about 85% of isolates from these neonatal infections. The *E. coli* K1 capsule is immunochemically identical to the capsular antigen of *N. meningitidis* group B. Fatality rates for infants with meningitis caused by *E. coli* K1 are higher than the fatality rates for infants infected with non-K1 strains. In addition to the neonatal population, *E. coli* remains a clinically significant isolate in blood cultures from adults. *E. coli* bacteremia in adults may result from a urogenital tract infection or from a GI source.

Uropathogenic *Escherichia coli*

E. coli is widely recognized as the most common cause of UTIs in humans. The *E. coli* strains that cause UTIs usually originate in the large intestine as resident biota and can exist either as the predominant *E. coli* population or as a small part of the *E. coli* strains in the large intestine. Strains causing lower UTIs and acute pyelonephritis in immunocompetent hosts are different from strains causing disease in the urinary tracts of individuals who are compromised either by urinary tract defects or by instrumentation such as placement of catheters. *E. coli* strains that cause acute pyelonephritis in immunocompetent hosts have been shown to be the dominant resident *E. coli* in the colon. They belong to a few serotypes and are resistant to the antibacterial activity of human serum. Conversely, isolates from immunocompromised hosts consist of a wide variety of strains.

Strains that cause UTIs produce factors that allow them to attach to the urinary epithelial mucosa. The primary virulence factor associated with the ability of *E. coli* to cause UTIs is the production of pili, which allow uropathogenic strains to adhere to epithelial cells and not be washed out with urine flow. For example, about 80% of *E. coli* isolates causing acute pyelonephritis express pyelonephritis-associated pili encoded by the *papA* and *papG* genes. Other factors contributing to the virulence of uropathogenic *E. coli* include flagella, cytolysins, and aerobactin. Flagella facilitate bacterial dissemination. Cytolysins, also often characterized as hemolysins, can kill immune effector cells and inhibit phagocytosis and chemotaxis of certain white blood cells. Aerobactin allows the bacterial cell to chelate iron; free iron is generally unavailable within the host for use by bacteria.

Gastrointestinal *Escherichia coli* infections

E. coli produces several different GI syndromes and causes an estimated 10% of all case of foodborne, bacterial, acute gastroenteritis in the United States. Based on virulence factors, clinical manifestation, epidemiology, and different O and H serotypes, there are five major categories, or pathotypes, of diarrheagenic *E. coli*: **enterotoxigenic *Escherichia coli* (ETEC)**, **enteroinvasive *Escherichia coli* (EIEC)**, **enteropathogenic *Escherichia coli* (EPEC)**, **Shiga toxin-producing *Escherichia coli* (STEC)**, and **enteroaggregative *Escherichia coli* (EAEC)**. These five categories are sometimes collectively referred to as enterovirulent *E. coli* or diarrheagenic *E. coli*. The serotypes associated with these categories and the features associated with the intestinal infections produced by these strains are summarized in Table 19.4. It is often difficult to distinguish among *E. coli* strains that are part of the normal microbiota and the diarrheagenic strains by culture.

Enterotoxigenic *Escherichia coli*

ETEC strains are associated with diarrhea in adults and especially children living in tropical and subtropical climates, particularly in developing countries, where it is one of the major causes of infant bacterial diarrhea. In the United States and other Western industrialized nations, ETEC diarrhea is the most common cause of diarrheal disease in travelers, sometimes referred to as **traveler's diarrhea**. Travelers from industrialized countries often become infected with ETEC when visiting developing nations. ETEC infection is spread commonly via consumption of contaminated food or water. Poor hygiene, reduced availability of potable water, and inadequate sanitation are major contributing factors in the spread and transmission of this disease. A high infective dose (10^6 to 10^{10} organisms) is necessary to initiate disease in an immunocompetent individual. Protective mechanisms such as stomach acidity have been noted as inhibiting colonization and initiation of disease; *achlorhydria*, deficiency of hydrochloric acid within the stomach, seems to be a high-risk factor.

Colonization of ETEC in the proximal small intestine is mediated by greater than 20 fimbriae and nonfimbrial adhesion factors that permit ETEC to bind to specific receptors on the intestinal microvilli. Once ETEC strains are established, they can release one or both of two toxins: heat-labile toxin (LT) or heat-stable toxin (ST). The LT is similar in action and amino acid sequence to cholera toxin from *Vibrio cholerae*. LT consists of two fragments (A and B) that follow the A/B model of bacterial toxins in which A is the enzymatically active portion. The B moiety, or binding portion, confers the specificity. The B portion binds to the GM_1 ganglioside of the intestinal mucosa, providing entry for the A portion.

The A portion activates cellular adenylate cyclase, causing an increase in the conversion of adenosine triphosphate to cyclic adenosine monophosphate (cAMP). The consequence of cAMP accumulation is hypersecretion of electrolytes and fluids into the intestinal lumen, resulting in watery diarrhea similar to cholera. In contrast, ST stimulates guanylate cyclase, causing increased production of cyclic guanosine monophosphate, accumulation of which also causes hypersecretion.

The usually mild, self-limiting disease caused by ETEC is characterized by watery diarrhea, abdominal cramps, and sometimes nausea, usually with no vomiting or fever.

Table 19.4 Features of pathogenic *Escherichia coli*

Type	Virulence factors	Relevant disease	Relevant serotypes	Laboratory tests
Uropathogenic <i>E. coli</i>				
UPEC	P pilus/ <i>pap</i> pili, type 1 fimbriae	UTIs		
DAEC ^a	Afa/Dr adhesions	UTIs		
Enteric pathogens				
EPEC	Type IV bundle-forming pilus, type III secretion of Tir	Infantile diarrhea	O55:NM O55:H6 O111:NM O111:H2 O114:NM O114:H2	HeLa cell adherence assay, DNA probes
EHEC	Shiga toxins/verotoxin	Hemorrhagic diarrhea, colitis, HUS	O157:H7 O157:NM O26:H11 O104:H21 O111:H2 O111:H8 O113:H21 O118:H2	SMAC plates, MUG
EIEC	Invasion plasmid antigen	Dysentery	O124:H30 O143:NM O164:NM	DNA probes
ETEC	LT, ST	Traveler's diarrhea	O6:NM O6:H16 O8:H9 O25:NM O27:NM O63:H12	Immunoassays for LT or ST
Enteroadherent <i>E. coli</i>				
EAEC	AAF Afa/Dr adhesions, AIDA-1, EAST, PET	Persistent pediatric diarrhea	O44:H18	
DAEC ^a	Afa/Dr adhesions	Pediatric diarrhea, UTIs		HeLa cell adherence assay, DNA probes
Extraintestinal pathogens				
	Capsule	Septicemia and meningitis	K1	

^aDAEC causes both UTIs and gastrointestinal infections.

AAF, Aggregative adherence fimbriae; Afa/Dr, afimbrial adhesins; AIDA-1, adhesin involved in diffuse adherence; DAEC, diffusely adherent *E. coli*; EAEC, enteroaggregative *E. coli*; EHEC, enterohemorrhagic *E. coli*; EIEC, enteroinvasive *E. coli*; EPEC, enteropathogenic *E. coli*; EAST, enteroaggregative heat stable enterotoxin; ETEC, enterotoxigenic *E. coli*; HUS, hemolytic uremic syndrome; LT, labile toxin; MUG, 4-methylumbelliferyl β-D-glucuronide; NM, nonmotile; PET, plasmid-encoded toxin; SMAC, MacConkey agar containing sorbitol; ST, stable toxin; UPEC, uropathogenic *E. coli*; UTI, urinary tract infection.

Mucosal penetration and invasion do not appear to be part of ETEC disease. ETEC infections must be differentiated from other diarrheal illnesses that can appear similar.

Diagnosis of ETEC infection is made primarily by the patient's travel history, characteristic symptoms, and the isolation of solely lactose-fermenting organisms on differential

media in stool cultures. Immunologic assays to detect the two toxins from culture supernatants are commercially available. Multiplex PCR performed on stool samples to detect different diarrheagenic strains of *E. coli* are also available. For ETEC, the targets are the plasmid-borne *eltA* and *eltB* genes, which code for the A and B subunits of LT, and the *est* gene, which codes for ST.

Enteropathogenic *Escherichia coli*

EPEC was the first diarrheagenic *E. coli* described. It was identified as a cause of infantile diarrhea in 1945, but it was not until the late 1960s and 1970s that certain O serogroups of EPEC were linked to these isolates. However, only certain H antigenic types within each O serogroup were connected to the intestinal infections, and O serogrouping could not differentiate these *E. coli* strains from strains of normal biota. In 1978, Levine and colleagues attempted to settle the dispute concerning the pathogenic role of EPEC by challenging volunteers with EPEC strains that lacked the toxins of ETEC and the invasiveness of EIEC. The study showed that these EPEC strains caused distinct diarrhea. Subsequent studies showed the adhesive property of EPEC strains, a characteristic not seen in ETEC or EIEC strains. The virulence of EPEC is defined as producing attaching and effacing lesions on the intestinal epithelium but inability to produce Shiga toxins and LT or ST enterotoxins.

Diarrheal outbreaks caused by EPEC have occurred in hospital nurseries and daycare centers, but cases in adults are rarely seen. The infective dose is high, typically 10^8 to 10^{10} viable cells. The illness is characterized by low-grade fever, malaise, and vomiting, with persistent watery diarrhea, typically in children younger than 2 years of age. The stool typically contains large amounts of mucus, but apparent blood is not present. Detection of diarrheal illness attributable to EPEC depends primarily on the suspicion of the physician. In cases of severe diarrhea in children younger than 1 year of age, infection with EPEC should be suspected. These strains have a characteristic pattern of adherence to human epithelial-2 (HEp-2) cell cultures. Serologic typing with pooled antisera may be performed to identify EPEC serotypes, but this is generally used for epidemiologic studies rather than for diagnostic purposes. Diagnosis by nucleic acid amplification tests (NAATs) is based on detection of the *bfpA* gene, which codes for type IV bundle-forming pilus.

Enteroinvasive *Escherichia coli*

EIEC infection is rare in the United States and seen less commonly in developing countries than ETEC or EPEC. EIEC differs greatly from EPEC and ETEC strains. Enteroinvasive strains genetically and phenotypically resemble *Shigella* spp. and, like *Shigella*, produce dysentery with direct penetration, invasion, and destruction of the intestinal mucosa. The diarrheal illness resembles that produced by *Shigella*, although it tends to be milder, which likely leads to underreporting. EIEC is less invasive than *Shigella* spp., and the infective dose is 1000 to 10,000 times greater for EIEC. Direct transmission of EIEC from person to person via the fecal-oral route has been reported. EIEC infections seem to occur in adults and children alike. The clinical infection is characterized by fever, severe abdominal cramps, malaise, and bloody diarrhea.

The organisms might be easily misidentified because of their similarity to shigellae. EIEC strains can be nonmotile and generally do not ferment lactose; cross-reaction between shigellae and EIEC O antigens has been reported. EIEC isolates can be mistaken for nonpathogenic *E. coli*. Although EIEC does not decarboxylate lysine, more than 80% of *E. coli* strains do decarboxylate lysine. Misidentification might be another reason for cases of diarrheal illness resulting from EIEC being underreported.

The enteroinvasiveness of EIEC must be demonstrated for definitive identification. The tests currently available to determine the invasive property of EIEC are not performed in most clinical microbiology laboratories. It is possible to detect invasiveness using monolayer HEp-2 cell cultures or with NAATs to detect invasion genes. These assays detect the *ipaH* gene, which codes for the invasion plasmid antigen H. However, both EIEC and *Shigella* spp. express this gene. These assays are used to screen stool samples, eliminating the need for other tests to identify EIEC.

Shiga toxin-producing *Escherichia coli*

STEC refers to *E. coli* strains that produce **Shiga toxin 1** (Stx1) and/or Shiga toxin 2 (Stx2). The toxins are so named because of their similarity to the Shiga toxin produced by *Shigella dysenteriae* type 1. Stx is also referred to as verotoxin because this cytotoxin produces damage to Vero cells (African green monkey kidney cells)—hence the term *verotoxin*. Stx1 and Stx2 are immunologically distinct, and the genes, *stx1* and *stx2*, are carried on a phage that has integrated into the bacterial chromosome. Stx follows the A/B model of bacterial toxins.

Shiga toxins preferentially affect endothelial cells of the GI tract and renal glomeruli. In 1982, the O157:H7 strain of *E. coli* was first recognized during an outbreak of hemorrhagic diarrhea and colitis. These isolates become what is referred to as enterohemorrhagic *E. coli* (EHEC). The EHEC strain serotype O157 has since been associated with hemorrhagic diarrhea, colitis, and hemolytic uremic syndrome (HUS). HUS is characterized by low platelet count, hemolytic anemia, and kidney failure. EHEC is a subset of STEC that causes HUS.

The classic illness caused by EHEC produces a watery diarrhea that progresses to bloody diarrhea with abdominal cramps and low-grade fever or an absence of fever. However, symptoms can be variable from mild to life-threatening HUS. Stool specimens contain no leukocytes, which distinguishes it from dysentery caused by *Shigella* spp. or EIEC infections. ETEC infection is potentially fatal, especially in young children and older adults in nursing homes. Processed meats such as undercooked ground beef (40% of outbreaks), unpasteurized dairy products and apple cider, bean sprouts, and spinach all have been implicated in the spread of infection. In 2019, consumption of contaminated romaine lettuce was linked to a 27-state outbreak involving at least 167 people. Of reported patients, 85 were hospitalized and 15 developed HUS; there were no deaths.

Stx1 and Stx2 have been linked to over 250 serotypes. However, STEC O157 accounts for up to 80% of STEC infections worldwide and has a hospitalization rate of 17% to 46%. The infective dose for STEC O157 infection is extremely low, 50 to 200 colony-forming units. This likely explains its high incidence. Any of the STEC serotypes can cause clinical syndromes like that produced by STEC O157. In the United States, 265,000 cases of STEC are reported annually, and about 36% are STEC O157. See [Table 19.4](#) for a list of non-O157 STEC isolated from patients with bloody diarrhea, hemorrhagic colitis, or HUS.

In the laboratory, STEC can be identified by one of three methods:

- Stool culture on selective and highly differential medium, with subsequent serotyping
- Detection of the Shiga toxin in stool filtrates

- Demonstration of a fourfold or greater increase in Shiga toxin–neutralizing antibody titer

Current guidelines recommend that all stool specimens submitted for routine testing of enteric pathogens also include culture and toxin detection to identify STEC.

Stool culture procedures for STEC should incorporate MAC agar containing sorbitol (SMAC) instead of lactose, or SMAC with cefixime and tellurite (SMAC-CT). However, some strains of STEC are sensitive to tellurite. *E. coli* O157 does not ferment sorbitol in 24 hours, a characteristic that differentiates it from most other *E. coli* strains. STEC O157 appears colorless on SMAC agar, whereas most other strains produce pink colonies from sorbitol fermentation. The use of this differential medium facilitates the primary screening of STEC O157. Although isolation of other non-sorbitol-fermenting organisms may occur in 15% of cultures, STEC O157 produces heavy growth when present. An emergent phenotype, the sorbitol-fermenting, nonmotile *E. coli* O157:NM (NM indicates nonmotile) has been seen in European outbreaks, so relying on sorbitol as a single test to identify O157 strains is unwise. The use of CHROMagar O157 (Becton Dickinson, Franklin Lakes, NJ) has increased the recovery of ETEC O157 from stool specimens and is recommended over nonselective media. This medium has a proprietary formulation resulting in STEC O157 strains producing mauve-colored colonies.

In addition to sorbitol fermentation, the commercially available 4-methylumbelliferyl- β -D-glucuronide (MUG) assay is a biochemical test used to screen isolates for *E. coli* O157:H7. *E. coli* O157:H7 rarely produces the enzyme β -glucuronidase, whereas 92% of other strains do produce it. If the enzyme is present, MUG is cleaved, and a fluorescent product is formed. Sorbitol-negative and MUG-negative colonies are subsequently subcultured for serotyping using *E. coli* O157:H7 antiserum.

Enzyme-linked immunosorbent assay (ELISA) or latex agglutination can be used to detect the O157 antigen on culture isolates. In the latex agglutination test, isolates must be tested with the negative control to detect nonspecific agglutination. The O157 somatic antigen, which is usually the target in the commercial assays, can present a problem with regard to specificity because other enteric bacteria produce false-positive results. It is also important to confirm biochemically the identification of MUG-negative or sorbitol-negative colonies as *E. coli* isolates.

Testing for flagella antigens might require subculturing the bacteria several times. When colonies are taken directly from the SMAC plate, the test for the H7 antigen may be initially negative. It is helpful to grow these isolates in motility media first to enhance flagella production and agglutination with the latex particles. Approximately 85% of *E. coli* O157 isolates from humans possess H7, whereas 12% are nonmotile.

After serotyping, isolates may be tested for the presence of Stx or *stx* genes. Reports have shown that all *E. coli* O157:H7 strains produce high levels of cytotoxins, and STEC strains may be detected using cell culture assays with Vero cells. Because other toxins present in diarrhea stools can produce similar cytopathic effects, this test must be verified with specific antitoxins to Stx1 and Stx2.

If no EHEC colonies are identified, it is recommended that a loopful of bacteria (sweep) taken from the least selective agar plate be tested for Shiga toxin by enzyme immunoassay

(EIA) or NAAT. If the sweep is positive for Shiga toxin, individual colonies should then be tested. If the sweep is negative for Shiga toxin, the specimen is reported negative for STEC.

Patients with hemorrhagic colitis shed the organisms for only brief periods; nevertheless, Shiga toxins may still be detected in the stool. Free Shiga toxin present in stool specimens can be directly detected by EIAs in samples that yield negative culture results. The use of broth enrichment can increase the sensitivity of EIA testing. A portion of the stool sample should be placed into an enrichment broth. Gram-negative (GN) broth is commonly used. The broth should be incubated overnight at 35°C, and an EIA can be used to detect Stx. Even if stools are screened for Stx and cultured for STEC, as recommended, underreporting of STEC likely occurs.

Molecular diagnostic tests such as the BD MAX Enteric Panel (Becton Dickinson), Prodesse ProGastro SSCS assay (Hologic, San Diego, CA), Biofire Filmarray GI Panel (bioMérieux, Durham, NC), and Shiga Toxin Direct Test (Great Basin Scientific, Salt Lake City, UT) offer increased sensitivity and faster turnaround times compared with culture and immunoassay for detecting STEC strains. Multiplex molecular panels detect the *stx1* and *stx2* genes.

Enterohemorrhagic *Escherichia coli*

EAEC cells stick to each other, cultured cell monolayers, plastic surfaces, and the surface of the intestinal mucosa. They adhere packed in an aggregative “stacked-brick” pattern on the cells and between the cells by means of fimbriae and can lead to biofilm formation. These organisms produce watery diarrhea, vomiting, dehydration, and occasionally abdominal pain, mostly in children. White blood cells and red blood cells are often absent from the stool. The symptoms often persist for at least 2 or more weeks.

EAEC may be an important cause of diarrhea in infants in the United States and other countries. It should also be considered a cause of diarrhea in patients with human immunodeficiency virus (HIV) infection. In some regions, despite a high infective dose (10^8 to 10^{10}), EAEC symptomatic cases exceed ETEC cases. In 2011, an outbreak in Europe caused by EHEC (O104:H4) that also had virulence related to EAEC resulted in more than 3400 total infections and about 850 cases of HUS with 32 HUS-related deaths. Most of the cases occurred in Germany, and trace-back studies indicated that fresh sprouts produced by a farm in Lower Saxony were responsible for the outbreak. At least two cases were reported in the United States in individuals who had recently traveled to Germany.

The stacked-brick growth pattern on monolayer cell cultures is suggestive of EAEC; however, DNA probes for virulence genes offer a definitive identification. Isolates containing the *aggR* gene, a transcription regulator residing on a plasmid that activates several genes, are called typical EAEC. This gene is the target for molecular identification. EAEC also contains several toxin-encoding genes, on plasmids and the chromosome, that can be found on other diarrheagenic *E. coli* strains.

Other *Escherichia* species

There are currently seven recognized species within the genus *Escherichia*. Apart from *E. coli*, two other species are occasionally associated with human disease. *Escherichia fergusonii* has

been isolated from urine, blood, wounds, feces, and the gallbladder. *Escherichia albertii* is associated with diarrheal disease. Some *E. albertii* isolates carry the *stx2f* gene, which codes for a Shiga-like toxin, and the *cdtB* gene, which codes for a toxin found in *Clostridioides difficile*. These isolates can cause a condition resembling HUS.

Klebsiella

The genera *Klebsiella* can be found in the intestinal tract of humans and other animals as normal microbiota, or are free-living in soil, water, and on plants. These microorganisms have been associated with various opportunistic and health-care-associated infections, particularly pneumonia, wound infections, and UTIs. Members of these genera demonstrate variable biochemical reactions. Common characteristics include:

- Most grow on Simmons citrate and in potassium cyanide (KCN) broth.
- None produce H₂S.
- A few hydrolyze urea slowly.
- All are methyl red test negative and Voges-Proskauer positive.
- With a few exceptions, no indole is produced from tryptophan.
- Motility is variable.

The genus *Klebsiella* comprises several species, including *K. aerogenes*, *K. michiganensis*, *K. oxytoca*, *K. pneumoniae* subsp. *ozaenae*, *K. pneumoniae* subsp. *pneumoniae*, *K. pneumoniae* subsp. *rhinoscleromatis*, and *K. variicola*.

With the exception of *K. aerogenes*, the absence of motility distinguishes *Klebsiella* spp. from most other members of the family Enterobacteriaceae. Differential features of *Klebsiella* spp. are shown in Table 19.5. *K. pneumoniae* subsp. *pneumoniae* is the most commonly isolated and has the distinct feature of possessing a large polysaccharide capsule. The capsule offers the organism protection against phagocytosis and antimicrobial absorption, contributing to its virulence. The capsule is also responsible for the moist, mucoid colonies characteristic of *K. pneumoniae*. Occasionally evident in direct smears from clinical materials, this capsule is sometimes helpful in providing a presumptive identification. Fig. 19.2 illustrates the mucoid appearance of *K. pneumoniae* on MAC agar.

Colonization of gram-negative bacilli in the respiratory tracts of hospitalized patients, particularly by *K. pneumoniae* subsp. *pneumoniae*, increases with the length of hospital stay. *K. pneumoniae* subsp. *pneumoniae* is a frequent cause of lower respiratory tract infections among hospitalized patients and in immunocompromised hosts such as newborns, older adult patients, and seriously ill patients on respirators. Carbapenemase-producing *K. pneumoniae* is an important cause of ventilator-associated pneumonia. Patients with rectal and tracheal colonization of carbapenemase-producing *K. pneumoniae* receiving prolonged antimicrobial therapy for non-carbapenemase-producing *K. pneumoniae* infection are at increased risk for infection.

Other infections commonly associated with *K. pneumoniae* subsp. *pneumoniae* involving immunocompromised hosts are wound infections, UTIs, liver abscesses, and bacteremia. Reports describe hospital-acquired outbreaks of *Klebsiella* resistant to multiple antimicrobial agents in newborn nurseries. These outbreaks have been attributed to the plasmid transfer

Table 19.5 Differentiation of common species within the genus *Klebsiella*

Test or substrate	<i>K. pneumoniae</i> subsp. <i>pneumoniae</i>			<i>K. oxytoca</i>			<i>K. pneumoniae</i> subsp. <i>ozaenae</i>		
	Sign	% +	(% +)	Sign	% +	(% +)	Sign	% +	(% +)
Urease	+	95.4	(0.1)	+	90		d	0	(14.8)
Indole	–	0		+	99		–	0	
Methyl red	– or +	10		–	20		+	97.7	
Voges-Proskauer	+	98		+	96		–	0	
Citrate (Simmons)	+	98	(0.6)	+	95		d	30	(32.4)
Gelatin (22° C)	–	0	(0.2)	–	0		–	0	
Lysine decarboxylase	+	98	(0.1)	+	99		– or +	40	(6.3)
Malonate	+	92.5		+	98		–	6	
Mucate	+	90		+	93		– or +	25	
Sodium alginate (utilization)	+ or (+)	88.5	(9.2)	nd			– or (+)	0	(11)
Gas from glucose	+	96		+	97		d	50	(9.4)
Lactose	+	98.7	(1)	+	100		d	30	(61.3)
Dulcitol	– or +	30		+ or –	55		–	0	
Organic acid media									
Citrate	+ or –	64.4		nd			– or +	18	
D-Tartrate	+ or –	67.1		nd			– or +	39	

+, ≥90% positive within 1 or 2 days; (+), positive reaction after ≥3 days (decarboxylase tests: 3 or 4 days); –, ≥90% no reaction in 30 days; + or –, most cultures positive, some strains negative; – or +, most strains negative, some cultures positive; d, different reactions; +, (+), –, nd, no data.

Modified from Ewing, W. H. (1986). *Edwards and Ewing's identification of enterobacteriaceae* (4th ed.). East Norwalk, CT: Appleton & Lange.

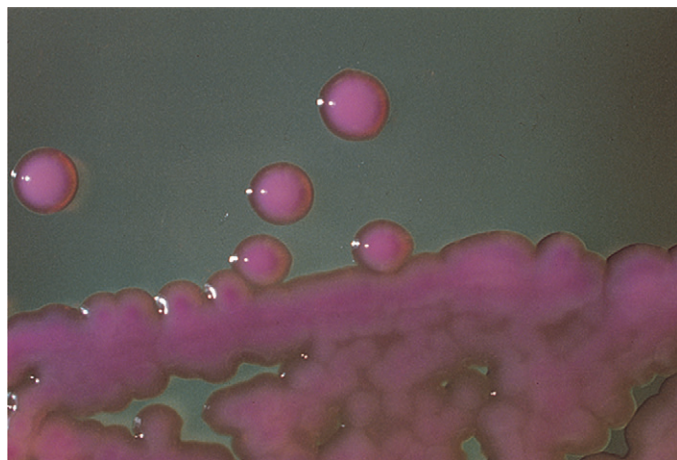


Fig. 19.2 Mucoid appearance of *Klebsiella pneumoniae* on MacConkey agar.

of antimicrobial resistance. Although antimicrobial resistance has been increasing within the family Enterobacteriaceae, it is probably most severe with *K. pneumoniae* because of the presence of the *K. pneumoniae* carbapenemase.

Other *Klebsiella* spp. have been associated with numerous infections. *K. pneumoniae* subsp. *ozaenae* produces infections resembling those caused by *K. pneumoniae* subsp. *pneumoniae*. In addition, isolates have been linked to antimicrobial-associated hemorrhagic colitis. *K. pneumoniae* subsp. *ozaenae* has been isolated from nasal secretions and cerebral abscesses. This organism causes atrophic rhinitis, a tissue-destructive disease restricted to the nose. Biochemically, *K. pneumoniae* subsp. *ozaenae* is identical to *K. pneumoniae* subsp. *pneumoniae* except for its production of indole, and there are reports of ornithine-positive isolates as well. *K. pneumoniae* subsp. *ozaenae* is highly associated with the presence of plasmid-mediated ESBLs, contributing to the large numbers of antimicrobial-resistant hospital-acquired infections seen today.

K. pneumoniae subsp. *rhinoscleromatis* has been isolated from patients with rhinoscleroma, an infection of the nasal cavity that manifests as an intense swelling and malformation of the entire face and neck. Cases of rhinoscleroma have been reported in Africa and South America. This subspecies is not isolated from the environment or GI tract and is more commonly seen in tropical regions. Although it is relatively easy to differentiate among the *K. pneumoniae* subspecies with biochemical testing, MALDI-TOF mass spectrometry might not be able to do so.

K. aerogenes (formerly *Enterobacter aerogenes*) has been known to cause healthcare-associated infections such as respiratory infections, UTIs, sepsis, and post-neurosurgical meningitis, particularly in severely ill patients in intensive care units. *K. aerogenes* has also been linked to poor clinical outcomes in patients with bloodstream infections relative to those caused by *Enterobacter* spp. In recent years, *K. aerogenes* has become a significant multidrug-resistant pathogen. In particular, carbapenem-resistant *K. aerogenes* has been reported in several countries. Although colistin has been considered as a valuable therapeutic option, many reports of colistin-resistant *K. aerogenes* have been described during the past several years. The first identified plasmid-mediated colistin-resistant gene is the *mcr-1* gene, characterized as one of the main mechanisms of colistin resistance in *K. aerogenes*.

Phylogenetic studies resulted in the reclassification of some *Klebsiella* species into the genus *Raoultella*. These species, *Raoultella ornithinolytica* (indole and ornithine decarboxylase-positive), *Raoultella terrigena*, and *Raoultella planticola*, have been isolated rarely from the urine, respiratory tracts, and blood of humans.

Case check 19.1

The Case in Point illustrates typical predisposing risk factors for increased colonization of gram-negative bacilli in hospitalized patients. These include length of hospital stay, weakened immune response of older adult patients, and underlying serious illnesses, such as diabetes.

Enterobacter and Cronobacter

The genus *Enterobacter* is composed of at least 22 species. Many are found in the environment including soil, water, sewage, and vegetables. The *E. cloacae* complex consists of several (about a dozen) genetically similar, clinically relevant species that include *E. asburiae*, *E. cancerogenus*, *E. cloacae*, *E. ludwigii*, and *E. hormaechei*. These common healthcare-associated pathogens are of particular concern because of their increasing multidrug resistance. After *K. pneumoniae*, the *Enterobacter* spp. are the second most common carbapenem-resistant Enterobacteriaceae in the United States. *E. cloacae* and *E. hormaechei* are the two most common clinical isolates from this group and have been associated with pneumonia, wounds, UTIs, septicemia, and meningitis. Species have also been associated with contaminated medical devices.

Except for *E. asburiae*, members of this genus are motile. The colony morphology of many of the species resembles *Klebsiella* spp. when grown on MAC agar. *Enterobacter* spp. grow on Simmons citrate medium and in KCN broth; the methyl red test is negative, and the Voges-Proskauer test is positive. However, in contrast with *Klebsiella*, *Enterobacter* spp. usually produce ornithine decarboxylase; lysine decarboxylase is produced by most species but not by *E. asburiae* or *E. cloacae*. Distinguishing characteristics among *E. cloacae*, *E. hormaechei*, and *K. pneumoniae* are shown in Table 19.6. *E. asburiae* biochemically resembles *E. cloacae* and has been isolated from blood, urine, feces, sputum, and wounds. *E. cancerogenus* has been associated with osteomyelitis after traumatic wounds.

The genus *Cronobacter*, within the family Enterobacteriaceae, currently contains seven species. *C. sakazakii* and *C. malonaticus* are the most common human isolates. *C. sakazakii* has been documented as a pathogen in neonates causing meningitis, bacteremia, and necrotizing enterocolitis, often coming from powdered infant formula. It has also been isolated from cultures taken from brain abscesses and respiratory and wound infections. *C. sakazakii* typically produces a yellow pigment (Fig. 19.3). *C. malonaticus* is more often found in adults. Because of their biochemical similarities, it is difficult to identify cronobacters to the species level. The most accurate method is DNA sequencing of the *fusA* and *rpoB* genes.

Table 19.6 Diagnostic features of *Enterobacter cloacae*, *Enterobacter hormaechei*, and *Klebsiella pneumoniae* subsp. *pneumoniae*

Test or substrate	<i>E. cloacae</i>			<i>E. hormaechei</i>			<i>K. pneumoniae</i> subsp. <i>pneumoniae</i>		
	Sign	% +	(% +)	Sign	% +	(% +)	Sign	% +	(% +)
Urease	+ w or –	65		+	87		+	95.4	(0.1)
Motility	+	95		+	52		–	0	
Lysine decarboxylase	–	0		–	0		+	98	(6.3)
Arginine dihydrolase	+	97	(2)	+	78		–	0	
Ornithine decarboxylase	+	96	(1.3)	+	91		–	0	
Gelatin (22° C)	–	0		–	0		–	0	(0.2)
Adonitol, gas	– or +	21.7	(1.3)	–	0		d	84.4	(0.3)
Inositol									
Acid	d	13	(8)	–	0		+	97.2	(0.9)
Gas	–	4.1	(1.5)				+	92.5	(1.5)
D-tartrate, Jordan's	– or +	30		–	13		+	95	
Sodium alginate (utilization)	–	0					+ or (+)	88.9	(8.9)

+, ≥90% positive within 1 or 2 days; (+), positive reaction after ≥3 days (decarboxylase tests: 3 or 4 days); –, ≥90% no reaction in 30 days; + or –, most cultures positive, some strains negative; – or +, most strains negative, some cultures positive; d, different reactions; +, (+), –, + w, weakly positive reaction.

From the Centers for Disease Control and Prevention, Atlanta, GA, and modified from Ewing, W. H. (1986). *Edwards and Ewing's identification of enterobacteriaceae* (4th ed.). East Norwalk, CT: Appleton & Lange.



Fig. 19.3 Mucoid, yellow-pigmented colonies of *Cronobacter sakazakii* growing on brain heart infusion agar. (Courtesy Jean Barnishan.)

Citrobacter

The genus *Citrobacter* consists of at least 19 species. The citrobacters are considered inhabitants of the GI tract and are associated with healthcare-associated infections, most frequently UTIs. The species most often isolated are *C. freundii* complex (includes *C. freundii*, *C. braakii*, and *C. youngae*, among others) and *C. koseri*. Although it is a known extraintestinal pathogen, *C. freundii* can be isolated in diarrheal stool cultures. *C. freundii* has been associated with infectious diseases acquired in hospital settings, often in patients older than 65 years of age. UTIs, pneumonias, septicemia, and intraabdominal abscesses have been reported. In addition, *C. freundii* has been associated with endocarditis in intravenous drug abusers. One reported case of *C. freundii* endocarditis required aortic valve replacement when antimicrobial therapy failed.

Most *Citrobacter* spp. hydrolyze urea slowly and ferment lactose, producing colonies on MAC agar that resemble those of *E. coli*. Almost all species grow on Simmons citrate medium (hence the genus name) and give a positive methyl red test. Because most (80%) *C. freundii* produce H₂S and some strains (50%) fail to ferment lactose, the colony morphology of *C. freundii* on primary selective media can be mistaken for *Salmonella* when isolated from stool cultures. Because of the pathogenic potential, it is important to differentiate *C. freundii* from *Salmonella*. Differentiation can be accomplished by using a minimal number of biochemical tests, such as urea hydrolysis and lysine decarboxylase. Most (70%) *C. freundii* hydrolyze urea, but all fail to decarboxylate lysine, whereas *Salmonella* fails to hydrolyze urea, and most isolates decarboxylate lysine. Several species of *Citrobacter* are similar and difficult to differentiate biochemically; hence, they are reported as *C. freundii* complex.

C. koseri is a pathogen documented as the cause of nursery outbreaks of neonatal meningitis, pneumonia, and brain abscesses. *C. braakii* is a rare human pathogen associated with community-acquired infections including septicemia in a patient with cervical cancer. Because of the difficulty in biochemically separating this species from other *Citrobacter* spp., infections might be underreported. *C. amalonaticus* is frequently found in feces, but no evidence has been found that it is a causative agent of diarrhea. It has been isolated from sites of extraintestinal infections, such as blood and wounds. *C. gillenii* and *C. murliniae* have also been isolated from human specimens.

Case check 19.2

The organism described in the Case in Point showed morphologic and biochemical characteristics of *Citrobacter* spp. The utilization of citrate is a key feature observed in this genus. *Citrobacter koseri* is frequently isolated from older adult males and is associated with antimicrobial resistance.

Kluyvera

The genus *Kluyvera* contains five closely related species: *K. ascorbata*, *K. cryocrescens*, *K. georgiana*, *K. intermedia*, and *K. sichuanensis*. *K. intermedia* was formerly classified as *Enterobacter intermedius*. *Kluyvera* spp. have been found in respiratory, urine, CSF, and blood cultures. Most strains are nonpigmented, but occasional isolates may produce a red-dish-blue or violet pigment. All species resemble *E. coli* colonies growing on MAC agar. Fig. 19.4 illustrates the colony characteristics of *Kluyvera* spp. Cephalothin and carbenicillin disk susceptibility tests can separate the two species *K. cryocrescens* and *K. ascorbata*. *K. cryocrescens* shows large zones of inhibition, and *K. ascorbata* has small zones. In addition, *K. ascorbata* does not ferment glucose at 5° C, whereas *K. cryocrescens* ferments glucose at this temperature.

Plesiomonas

Because of phenotypic characteristics, the genus *Plesiomonas* was formerly in the family Vibrionaceae. Like the vibrios and aeromonads, these organisms are oxidase-positive, glucose-fermenting, facultatively anaerobic, gram-negative bacilli. However, *Plesiomonas* was moved to the family Enterobacteriaceae because phylogenetic studies revealed a closer relatedness to members of that family. Unlike the Enterobacterales, *Plesiomonas* does not have the ability to produce gas from glucose, it is the only oxidase-positive member, and it is susceptible to the compound O/129. *P. shigelloides* is the only species in this genus.

Plesiomonads are straight, gram-negative bacilli that occur singly, in pairs, or in short chains or filamentous forms. They do not form capsules and are motile by monotrichous or two to five lophotrichous flagella. The genera *Plesiomonas* and *Shigella* share biochemical and antigenic features, and plesiomonads often cross-agglutinate with *Shigella sonnei*, *S. dysenteriae*, and even *S. boydii* antisera, hence the species name *shigelloides*. However, *P. shigelloides* possess a much lower virulence potential than *Shigella*, with a low symptomatic carriage rate among humans. *Plesiomonas* can be serotyped by somatic O antigens and their flagellar H antigen. Some of the numerous serotypes are ubiquitous, and others are confined to certain regions only.

P. shigelloides is found in soil and aquatic environments, but because of intolerance to increased NaCl and a minimum growth temperature of 8° C, isolates are generally found only in the fresh and estuarine waters of tropical and subtropical climates. They are widely distributed among warm- and cold-blooded animals, including dogs, cats, pigs, vultures, snakes, lizards, fish, newts, and shellfish.

P. shigelloides has emerged as a potential cause of gastroenteritis in humans, most often after the consumption of undercooked seafood, in particular shellfish, or untreated water. At least three major clinical types of gastroenteritis are caused by *Plesiomonas*:

- The more common watery or secretory diarrhea
- A subacute or chronic disease that lasts from 14 days to 2 to 3 months
- A more invasive, dysenteric form that resembles colitis

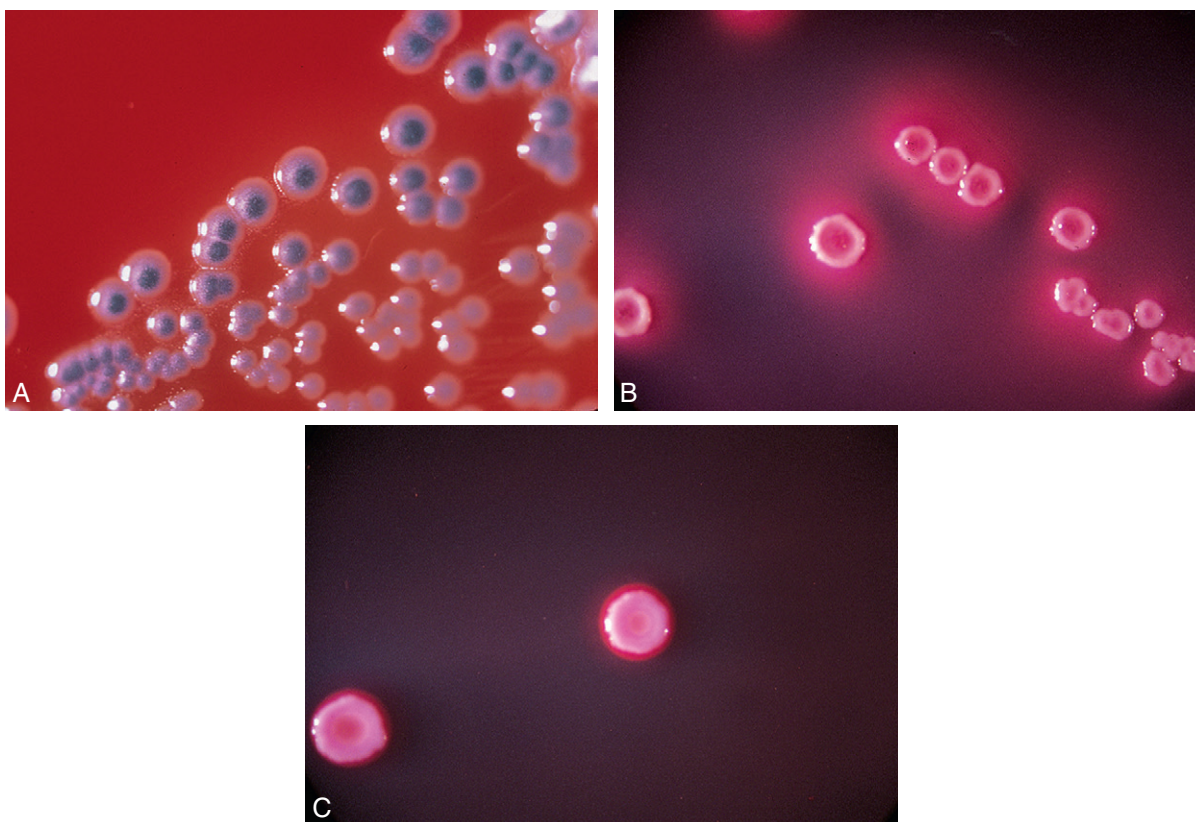


Fig. 19.4 A, Blue-violet pigment of *Kluyvera* spp. growing on sheep blood agar. This *Kluyvera* species resembles the colony morphology of *Escherichia coli* growing on MacConkey (MAC) agar. B, Appearance of *K. cryocrescens* growing on MAC agar. C, Appearance of *K. ascorbata* growing on MAC agar.

On average, 25% to 40% of symptomatic patients present with fever, vomiting, or both, and the single most common clinical symptom for all such patients is abdominal pain. Most cases are self-limiting, but antimicrobial therapy is indicated in severe and prolonged cases. Reports of *P. shigelloides* infection in patients with HIV infections are increasing, as are associations with inflammatory bowel disease.

Plesiomonads have also been isolated from several extraintestinal infections, most notably meningitis in neonates, septicemia, shock, and wounds. Occupational exposure can be a source of infections for veterinarians, zookeepers, aquaculturists, fish handlers, and athletes participating in water-related sports. More serious infections, such as bacteremia and meningitis, usually occur only in severely immunocompromised patients or neonates. Reports include cases of continuous ambulatory, peritoneal dialysis–associated peritonitis. Furthermore, biliary tract disease has been identified as a possible risk factor for bacteremia. Cases are possibly underreported because of the similarity to *E. coli* grown on most ordinary enteric media.

Antimicrobial therapy is indicated for patients with severe or chronic disease. Similarly, extraintestinal infections, particularly among neonates, often require antimicrobial therapy. Studies have shown a general resistance to the penicillin class of antibiotics, but penicillins combined with a β -lactamase inhibitor, as well as trimethoprim-sulfamethoxazole, are active. There have been reports of resistance to more than one aminoglycoside (e.g., gentamicin, tobramycin, amikacin), but the quinolones, azithromycin, cephalosporins, and carbapenems appear to be effective therapy.

Erwiniaceae

Pantoea

Pantoea is a diverse group of about two dozen species of yellow-pigmented organisms containing many plant pathogens. Some current members of the genus *Pantoea* were previously classified in the genus *Erwinia*. Current members of the genus *Erwinia* are environmental isolates and potential plant pathogens rarely recovered in human specimens. However, several strains have been isolated from clinical specimens including blood, CSF, sputum, and urine. *Pantoea agglomerans* gained notoriety with a nationwide outbreak of septicemia resulting from contaminated intravenous fluids. Originally designated as *Enterobacter agglomerans* complex, it includes lysine-, ornithine-, and arginine-negative or “triple decarboxylase-negative” members. Fig. 19.5 depicts a yellow-pigmented *P. agglomerans*.

Morganellaceae

Proteus

The genera *Proteus*, *Morganella*, and *Providencia* belong to the family Morganellaceae. They are widely disseminated in the environment, are normal intestinal microbiota, and are recognized as opportunistic pathogens. *Proteus mirabilis* is the most common clinical isolate. The family is distinguished from the other members of the Enterobacterales by their ability to deaminate the amino acid phenylalanine. Virtually no other members of the Enterobacterales synthesize the required enzyme, phenylalanine deaminase. None of the members of this family ferment lactose.

The genus *Proteus* consists of at least 13 species. *P. mirabilis* and *P. vulgaris* are widely recognized human pathogens, with *P. mirabilis* being the more common isolate. Both species have been isolated from urine, wounds, and ear and bacteremic infections. *Proteus* spp. are responsible for 3% of all healthcare-associated infections in the United States, particularly UTIs. They ascend the urinary tract, causing infections in both the lower and upper urinary tract. They can infect the proximal kidney tubules and can cause acute glomerulonephritis, particularly in patients with urinary tract defects or catheterization. The urease activity of *P. mirabilis* results in an increased urine pH and can lead to struvite kidney stones (calculi).

P. mirabilis and *P. vulgaris* are easily identified in the clinical laboratory because of their characteristic colony morphology. Both species, particularly *P. mirabilis*, can produce “swarming” colonies on nonselective media, such as SBA (Fig. 19.6). The swarming characteristic is a result of a tightly regulated cycle

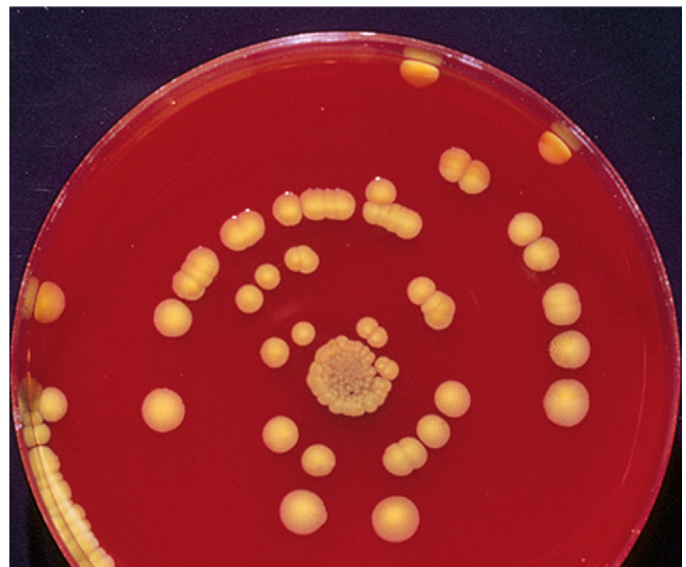


Fig. 19.5 Yellow-pigmented *Pantoea agglomerans* on sheep blood agar. (Courtesy Jean Barnishan.)

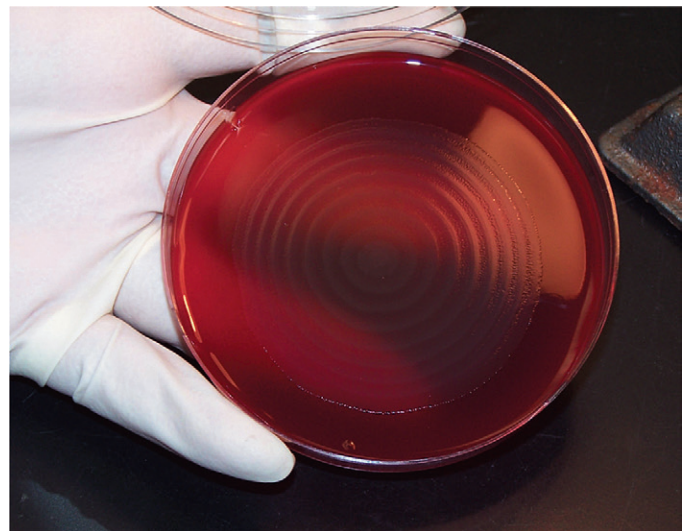


Fig. 19.6 Example of *Proteus mirabilis* swarming on sheep blood agar. (Courtesy Kimberly Walker and R. Abe Baalness.)

of differentiation from standard vegetative cells (swimmers) to hyperflagellated, elongated, polyploid cells (swarmers) capable of coordinated surface movement. Swarmer cells also produce the distinct odor associated with *Proteus* colonies, sometimes described as “burnt chocolate,” and are thought to play a role in the ascending nature of *Proteus*-associated UTIs.

Both species hydrolyze urea and produce H_2S , although some strains of *P. vulgaris* are negative for H_2S . *P. mirabilis* is differentiated from *P. vulgaris* by the indole and ornithine decarboxylase tests. *P. mirabilis* does not produce indole from tryptophan and is ornithine-positive, whereas *P. vulgaris* produces indole and is ornithine-negative. *P. vulgaris* ferments sucrose and gives an acid/acid reaction in triple sugar iron (TSI) agar. *P. penneri* can also swarm on nonselective media. *P. penneri* has been isolated from patients with diarrhea and UTIs, although the organism’s role in diarrheal disease has not been proven.

Morganella

The genus *Morganella* comprises one species of medical importance, *M. morganii*, with two subspecies, *M. morganii* subsp. *morganii* and *M. morganii* subsp. *sibonii*. Although not a common isolate, it is an important opportunistic pathogen because of its multidrug resistance and increased virulence. *M. morganii*, a recognized healthcare-associated pathogen, has been primarily identified as a cause of UTIs and occasionally wound infections and bacteremia. *M. morganii* subsp. *morganii* is differentiated from *M. morganii* subsp. *sibonii* by its inability to ferment trehalose. Most *M. morganii* subsp. *sibonii* are motile but do not swarm. Key identifying characteristics are listed in Table 19.7.

Providencia

The genus *Providencia* contains four clinically notable species: *P. alcalifaciens*, *P. stuartii*, *P. rettgeri*, and *P. rustigianii*. *P. rettgeri* is a documented pathogen of the urinary tract and has caused occasional outbreaks in health care settings. It has also been implicated in diarrheal disease among travelers. Similarly, *P. stuartii* has been implicated in outbreaks in burn units and has been isolated from urine cultures. Infections caused by *P. stuartii* and *P. rettgeri*, especially in immunocompromised patients, are particularly difficult to treat because of their resistance to antimicrobials. Studies of the epidemiology of *P. alcalifaciens* support its role in traveler’s diarrhea; however, its pathogenicity is not well understood. *P. rustigianii* is rarely isolated, and its pathogenicity also remains unproven. See Table 19.7 for the differentiating characteristics of medically important *Proteus*, *Providencia*, and *Morganella*.

Hafniaceae

Hafnia

The genus *Hafnia* is composed of three species: *H. alvei*, *H. paralvei*, and *H. psychrotolerans*. *Hafnia* spp. have been isolated from many anatomic sites in humans, animals, and in the environment. They can produce a verocytotoxin, although *H. alvei* is more likely to be toxigenic, and have been linked to gastroenteritis and are occasionally isolated in stool cultures. The species are differentiated based on malonate utilization, salicin and D-arabinose fermentation, and on β -glucosidase activity. A delayed positive citrate reaction is a major characteristic of *Hafnia*.

Table 19.7 Differentiating characteristics of selected species of *Proteus*, *Providencia*, and *Morganella*

Test	<i>Proteus penneri</i>	<i>Proteus mirabilis</i>	<i>Proteus vulgaris</i>	<i>Providencia alcalifaciens</i>	<i>Providencia stuartii</i>	<i>Providencia rettgeri</i>	<i>Morganella morganii</i>
Indole	–	+	+	+	+	+	+
Methyl red	+	+	+	+	+	+	+
Voges-Proskauer	–	– or +	–	–	–	–	–
Simmons citrate	–	+	d	+	+	+	–
Christensen urea	+	+	+	–	– or +	+	+
H_2S (TSI)	– (70%)	+	+	–	–	–	–
Ornithine decarboxylase	–	+	–	–	–	–	+
Phenylalanine deaminase	+	+	+	+	+	+	+
Acid produced from							
Sucrose	+	d	+	d	d	d	–
Mannitol	–	–	–	–	d	+	–
Salicin	–	–	d	–	–	d	–
Adonitol	–	–	–	+	–	+	–
Rhamnose	–	–	–	–	–	+	–
Maltose	+	–	+	–	–	–	–
Xylose	+	+	+	–	–	– or +	–
Arabitol	–	–	–	–	–	+	–
Swarms	+	+	+	–	–	–	–

+, $\geq 90\%$ positive reaction within 1 or 2 days; –, no reaction ($\geq 90\%$) in 30 days; – or +, most strains negative, some cultures positive; + or (+), most reactions occur within 1 or 2 days, some are delayed; + or –, most cultures positive, some strains negative; d, different reactions; H_2S , hydrogen sulfide; TSI, triple sugar iron agar.

Modified from Washington, J. (1981). *Laboratory procedures in clinical microbiology* (2nd ed.). New York: Springer-Verlag.

Edwardsiella

The genus *Edwardsiella* is composed of five species: *E. tarda*, *E. anguillarum*, *E. hoshinae*, *E. ictaluri*, and *E. piscicida*. *E. tarda* is the only recognized human pathogen. *E. tarda* is an opportunist, causing bacteremia, GI infections, and wound infections. It is reported to have a significant mortality rate. Patients with underlying hepatobiliary disease are at increased risk of infection. Members of this genus are negative for urea and positive for lysine decarboxylase, H_2S , and indole and do not grow on Simmons citrate.

Serratia

The genus *Serratia* is placed within the family Yersiniaceae, which also includes the genus *Yersinia*, considered a primary pathogen and is discussed later in the chapter. The genus *Serratia* comprises a diverse group of organisms with at least 25 named species. *Serratia* spp., widespread in the environment, are opportunistic pathogens isolated from outbreaks in health care settings. *S. marcescens*, *S. rubidaea*, and *S. plymuthica* are noted for producing a characteristic pink-to-red pigment, prodigiosin, especially when the cultures are incubated at room temperature. Pigment production is typically a characteristic in those strains of environmental origin. Fig. 19.7 illustrates the pigmentation of *S. marcescens* and *S. rubidaea*.

Clinically, *S. marcescens* is considered the most significant species, and many clinical isolates are nonpigmented. It has been found frequently in healthcare-associated infections of the urinary or respiratory tract and in bacteremic outbreaks in nurseries and cardiac surgery and burn units. Contamination of antiseptic solution used for joint injections resulted in an epidemic of septic arthritis. Ophthalmic infections caused by *S. marcescens* have also been reported.

S. odorifera, as the species name implies, emits a dirty, musty odor resembling that of rotten potatoes. It is isolated predominantly from sputum, blood, and urine. *S. liquefaciens*, *S. rubidaea*, *S. ficaria*, *S. fonticola*, and *S. quinivorans* have also been isolated from human sources.

Generally, *Serratia* spp. ferment lactose slowly and are positive for the ONPG test. They are differentiated from other members of the order by their ability to produce extracellular DNase. Like the *Klebsiella* and *Enterobacter* species, *Serratia* spp. are typically VP positive. *Serratia* spp. are also known for their resistance to a wide range of antimicrobials.

Susceptibility tests must be performed on each isolate to determine appropriate antimicrobial therapy.

Primary pathogens in the order Enterobacterales

Salmonella and *Shigella* are members of the family Enterobacteriaceae. These organisms produce GI illnesses in humans, and neither is considered normal biota of the human intestinal tract. *Salmonella* spp. can inhabit the GI tract of animals. Humans acquire the infection by ingesting the organisms in contaminated animal food products or insufficiently cooked poultry, milk, eggs, and dairy products. Animal reservoirs include poultry and rodents. Some *Salmonella* infections are transmitted by human carriers.

Infections caused by *Shigella* spp. are associated with human carriers responsible for spreading the disease; no animal reservoir has been identified. *Shigella* dysentery usually indicates improper sanitary conditions and poor personal hygiene. Infections caused by *Yersinia* spp., members of the family Yersiniaceae, are transmitted by a wide variety of wild and domestic animals. *Yersinia* infections include GI disease, mediastinal lymphadenitis, fulminant septicemia, and pneumonia.

Salmonella

Members of the genus *Salmonella* produce significant infections in humans and in certain animals. Other *Salmonella* serotypes are typically found in cold-blooded animals as well as in rodents and birds, which serve as their natural hosts. Annually, the CDC reports several outbreaks of human salmonellosis acquired from turtles and poultry. Salmonellae are gram-negative, facultatively anaerobic bacilli that morphologically resemble other enteric bacteria. On selective and differential media used primarily to isolate enteric pathogens (e.g., MAC), salmonellae produce clear, colorless, nonlactose-fermenting colonies; colonies with black centers are seen if the media (e.g., HE or XLD) contain indicators for H_2S production. The biochemical features for the genus include:

- Almost every isolate does not ferment lactose.
- Test results for indole, phenylalanine deaminase, urease, and the Voges-Proskauer test are negative.

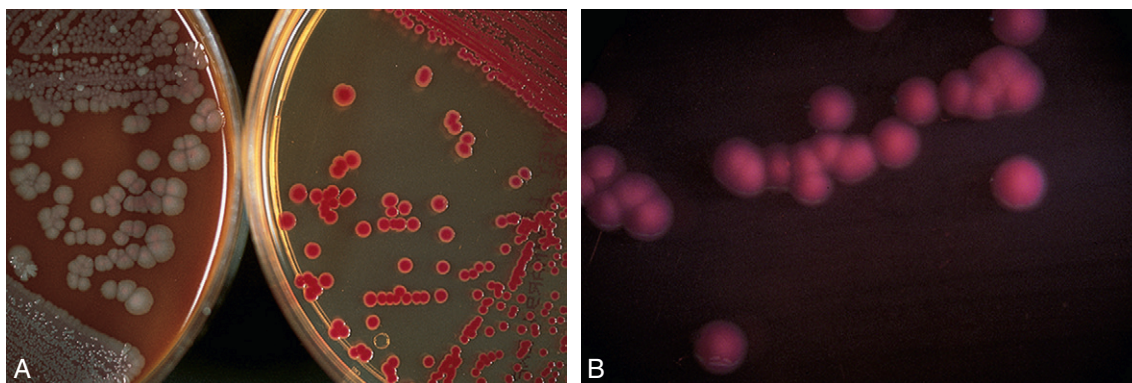


Fig. 19.7 **A**, *Serratia marcescens* growing on chocolate agar (left) and showing brick-red pigment when grown on MacConkey (MAC) agar (right). **B**, Pinkish red pigmentation of *Serratia rubidaea* growing on MAC agar.

- Most isolates produce H_2S ; a major exception is *Salmonella* Paratyphi A, which does not produce H_2S .
- They do not grow in media containing KCN.

Classification

Currently, based on DNA homology and sequencing, the genus *Salmonella* comprises only two species. *S. enterica* is the type species of the genus and accounts for almost all human infections. *S. bongori* is a rarely isolated species that is named after the town of Bongor in Chad, Africa. It was initially isolated in 1966 from a lizard and is usually isolated from cold-blooded animals and the environment.

Within the species *S. enterica* are six subspecies: *S. enterica* subsp. *enterica* (subspecies I), *S. enterica* subsp. *salamae* (subspecies II), *S. enterica* subsp. *arizonae* (subspecies IIIa), *S. enterica* subsp. *diarizonae* (subspecies IIIb), *S. enterica* subsp. *houtenae* (subspecies IV), and *S. enterica* subsp. *indica* (subspecies VI). Nearly all former *Salmonella* spp. have been placed as serotypes below the level of *S. enterica* subsp. *enterica* (e.g., *S. enterica* subsp. *enterica* serotype Typhi), which is often more simply written as *Salmonella* Typhi (serotype is capitalized and not italicized). Currently, greater than 2500 serotypes are recognized, but fewer than 100 are associated with human infections. Table 19.8 shows the characteristic features of *Salmonella* serotype Typhi, *Salmonella* serotype Choleraesuis, and *Salmonella* serotype Paratyphi. Members of the former genus *Arizona*, now subspecies IIIa of *S. enterica*, are found in infections with symptoms identical to those of *Salmonella* infections and may be transmitted to humans from pet turtles, snakes, and fish.

Virulence factors

Factors responsible for the virulence of salmonellae include capsule production and fimbriae used in adherence in initiating intestinal infection. Other factors that contribute to the virulence of salmonellae is their ability to traverse intestinal mucosa and survive inside host cells. Also, enterotoxins produced by certain *Salmonella* strains that cause gastroenteritis have been implicated as a significant virulence factor.

Antigenic structures

Salmonellae possess antigens resembling antigens of other enterobacteria. The somatic O antigens and flagellar H antigens are the primary antigenic structures used in serologic grouping of salmonellae. A few strains may possess capsular (K) antigens, designated Vi antigen. The serologic identification of the Vi antigen is important in identifying *Salmonella* serotype Typhi. Fig. 19.8 shows the antigenic structures used in serologic grouping and their locations. The heat-stable O antigen of salmonellae, as is the case with other enteric bacteria, is the lipopolysaccharide located in the outer membrane of the cell wall. Many different O antigens are present among the subspecies of *Salmonella*; more than one O antigen can also be found in a particular strain. The O antigens are designated by Arabic numbers.

In contrast with the O antigens, flagellar antigen proteins are heat labile. The H antigens of salmonellae occur in one of two phases: phase 1, the specific phase, and phase 2, the nonspecific phase. Phase 1 flagellar antigens occur only in a few serotypes and determine the immunologic identity of the particular serotype. Phase 1 antigens agglutinate only with homologous antisera. Phase 2 flagellar antigens occur among several strains. Shared by numerous serotypes, phase 2 antigens react with heterologous antisera. The heat-labile Vi (from the term *virulence*) antigen is a surface polysaccharide capsular antigen found in *Salmonella* serotype Typhi and a few strains of *Salmonella* serotype Choleraesuis. The capsular antigen plays a significant role in preventing phagocytosis of the organism. The Vi antigen often blocks the O antigen during serologic typing but may be removed by heating.

Clinical infections

In humans, salmonellosis can occur in several forms:

- Acute gastroenteritis or food poisoning characterized by vomiting and diarrhea
- Typhoid fever, the most severe form of enteric fever, caused by *Salmonella* serotype Typhi, and enteric fevers caused by other *Salmonella* serotypes (e.g., *Salmonella* Paratyphi and *Salmonella* Choleraesuis)

Table 19.8 Biochemical differentiation of selected members of the genus *Salmonella*

Test	<i>S. serotype Choleraesuis</i>	<i>S. serotype Paratyphi</i>	<i>S. serotype Typhi</i>	Other ^a
Arabinose fermentation	—	+	—	+
Citrate utilization	V	—	—	+
Glucose gas production	+	+	—	+
H_2S (TSI)	V	—	+	+
Lysine decarboxylase	+	—	+	+
Ornithine decarboxylase	+	+	—	+
Rhamnose fermentation	+	+	—	+
Trehalose fermentation	—	+	+	+

^aTypical strains in serogroups A to E.

—, $\geq 90\%$ of strains positive; +, $\geq 90\%$ of strains positive; H_2S , hydrogen sulfide; TSI, triple sugar iron agar; V, 10% to 89% of strains positive.

Data from Farmer, J. J., et al. (2007). Enterobacteriaceae: introduction and identification. In P. R. Murray, et al. (Eds.), *Manual of clinical microbiology* (9th ed.). Washington, DC: ASM Press.

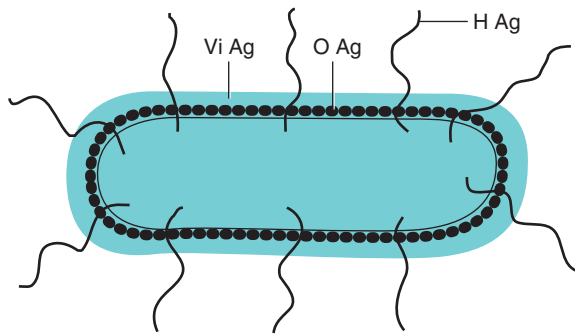


Fig. 19.8 Antigenic structures of salmonellae used in serologic typing. H Ag, flagellar antigen; O Ag, somatic O antigen; Vi Ag, masking (capsular) antigen.

- Nontyphoidal bacteremia and other extraintestinal complications
- Carrier state following *Salmonella* infection

Humans acquire the infection by ingesting the organisms in food, milk, and water contaminated with human or animal excreta. Except for *Salmonella* Typhi and *Salmonella* Paratyphi, salmonellae organisms infect various animals that serve as reservoirs and sources of human infections. *Salmonella* serotypes Typhi and Paratyphi have no known animal reservoirs, and infections seem to occur only in humans. Human carriers are often a source of these serotypes.

Gastroenteritis

Salmonellae are some of the most common causes of food poisoning. The CDC estimates the annual incidence of *Salmonella* infection to be 1.35 million. The *Salmonella* strains associated with gastroenteritis are usually those found in animals; most such strains in the United States are members of *S. enterica* subsp. *enterica*. The source of the infection is attributed primarily to poultry, milk, eggs, and egg products as well as to handling pets. Insufficiently cooked eggs and domestic fowl, such as chicken, turkey, and duck, are common sources of infection. More recently in the United States, there was a series of outbreaks by various *Salmonella* serotypes related to the ingestion of foodstuffs such as papayas, tahini, chicken, ground beef, eggs, turkey products, pasta salad, pre-cut melons, coconut, cereal, peaches, mushrooms, and onions. In 2015 to 2016, there were 907 cases resulting in 6 deaths linked to *Salmonella* Poona in 40 states. The outbreak was traced to cucumbers imported from Mexico and distributed to many states in the United States.

Numerous outbreaks also have been linked to calves and poultry at petting zoos. Handling live animals, such as reptiles, can also result in salmonellosis. In 2020, there was a 25-state outbreak of *Salmonella* Typhimurium associated with hedgehogs affecting 49 people. In addition, from August 2010 to June 2011, the CDC documented 109 cases of *Salmonella* Typhimurium from clinical and teaching laboratories. Infected individuals included students in microbiology teaching laboratories and employees in clinical microbiology laboratories and their household contacts.

Salmonella gastroenteritis occurs when enough organisms contaminate food that is maintained under inadequate refrigeration, allowing growth and multiplication of the organisms. The infective dose necessary to initiate the disease, approximately 10^6 bacteria, is much higher than the dose required

for shigellosis. Cooking utensils, such as knives, pans, and cutting boards used in preparing the contaminated food, can spread the bacteria to other food. Direct transmission from animal to person and from person to person in institutions has been reported.

The symptoms of intestinal salmonellosis, which may appear 8 to 36 hours after ingestion of contaminated food, include nausea, vomiting, fever and chills, diarrhea (watery or blood-tinged), and abdominal pain. Most cases of *Salmonella* gastroenteritis are self-limiting. Symptoms usually disappear within a few days, with few or no complications. Patients with sickle cell disease and other hemolytic disorders, ulcerative colitis, and malignancy seem to be more susceptible to salmonellosis. The infection can be more severe in very young children, older adults, and patients with other underlying disease. Dissemination can occasionally occur; in such cases, antimicrobial therapy is required.

The antimicrobials of choice include fluoroquinolone, azithromycin, and ceftriaxone. Nevertheless, susceptibility testing must be performed. Antimicrobial therapy is usually not indicated in uncomplicated cases. Antimicrobial therapy is believed to prolong the carrier state. Antidiarrheal agents are also restricted in cases of salmonellosis because these agents may encourage adherence and further invasion. In cases of dehydration, fluid replacement therapy may be indicated.

Enteric fevers

The clinical features of enteric fevers include:

- Prolonged fever
- Bacteremia
- Involvement of the reticuloendothelial system, particularly the liver, spleen, intestines, and mesentery
- Dissemination to multiple organs

Enteric fever caused by *Salmonella* Typhi is known as **typhoid fever**, a febrile disease that results from the ingestion of food contaminated with the organisms originating from infected individuals or carriers. *Salmonella* Typhi does not have a known animal reservoir; humans are the only known source of infection. Other enteric fevers include paratyphoid fevers, which may be caused by *Salmonella* serotypes Paratyphi A, B, and C and *Salmonella* serotype Choleraesuis. The clinical manifestations of paratyphoid fevers resemble typhoid fever but are less severe, and the fatality rate is lower.

Typhoid fever occurs more often in tropical and subtropical areas, where international travelers are more likely to acquire the infection. The highest incidences are found in Pakistan and India. Improper disposal of sewage, poor sanitation, and lack of a modern potable water system have caused outbreaks of typhoid fever when the organisms reach a water source. This situation is uncommon in the United States and other developed countries, where water is purified and treated, and handling of waste is standardized. Carriers, particularly food handlers, are important sources of infection anywhere in the world. Transmission through fomites is also possible. Laboratory workers in the microbiology laboratory have contracted typhoid fever while working with the organisms.

Typhoid fever develops approximately 7 to 14 days after ingestion of the organisms. The onset of symptoms depends on the number of organisms ingested; the larger the inoculum, the shorter the incubation period. Characteristically,

during the first week of the disease, the patient develops a sustained fever accompanied by malaise, anorexia, lethargy, myalgia, and a continuous dull frontal headache.

When the organisms are ingested, they can survive gastric acids and, on reaching the proximal end of the small intestine, they subsequently invade and penetrate the intestinal mucosa. At that time, the patient experiences constipation rather than diarrhea. The organisms gain entrance into the lymphatic system and are sustained in the mesenteric lymph nodes. They eventually reach the bloodstream and spread to the liver, spleen, and bone marrow, where they are immediately engulfed by mononuclear phagocytes. The organisms multiply intracellularly; later they are released into the bloodstream a second time. The febrile episode becomes more evident during this release of the organisms into the circulatory system. At this time, the organisms may be isolated easily from the blood. Fig. 19.9 shows the course of typhoid fever.

Without treatment, during the second and third weeks of the disease, the patient generally experiences sustained high fever with prolonged bacteremia. A maculopapular rash, described as “rose spots” (blanching, rose-colored papules), develops on the abdomen and extremities in up to 30% of cases. The organisms invade the gallbladder and Peyer patches of the bowel. They also reach the intestinal tract via the biliary tract. Involvement of biliary system sites initiates GI symptoms as the organisms reinfect the intestinal tract. The organism now exists in large numbers in the bowel and can be isolated from feces. The gallbladder becomes the foci of long-term carriage of the organism, occasionally reseeding the intestinal tract and shedding the organisms in the feces. Necrosis in the gallbladder leading to necrotizing cholecystitis and necrosis of the Peyer patches leading to hemorrhage and perforation of the bowel may occur as serious complications. Other complications that occur in typhoid fever include pneumonia, thrombophlebitis, meningitis, osteomyelitis, endocarditis, and abscesses.

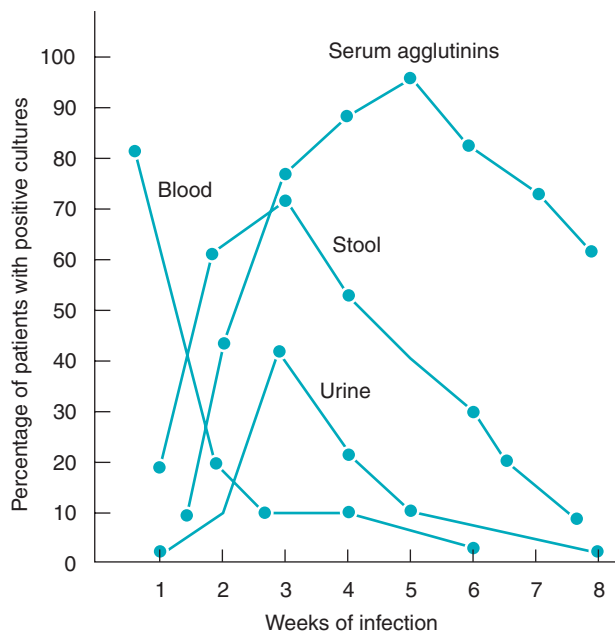


Fig. 19.9 Culture and serologic diagnosis of typhoid fever. (Modified from Procop, G. W., et al. (2017). *Koneman's color atlas and textbook of diagnostic microbiology* [7th ed.]. Philadelphia: Wolters Kluwer.)

Bacteremia

Salmonella bacteremia, with and without extraintestinal foci of infection caused by nontyphoidal *Salmonella*, is characterized primarily by prolonged fever and intermittent bacteremia. Many serotypes can produce bacteremia in patients with underlying health conditions such as acquired immunodeficiency syndrome, solid tumors, hematologic malignancies, and diabetes. The serotypes most associated with bacteremia in immunocompetent individuals are Typhimurium, Paratyphi, and Choleraesuis. The infection has been observed among two different groups: (1) young children, who experience fever and gastroenteritis with brief episodes of bacteremia, and (2) adults, who experience transient bacteremia during episodes of gastroenteritis or develop symptoms of septicemia without gastroenteritis. The latter manifestations were observed among patients who had underlying illnesses, such as malignancies and liver disease. The risk of metastatic complications, commonly endocarditis, could be more severe than the bacteremia itself, even in individuals who do not have underlying diseases. Cases of septic arthritis can also occur in patients who had asymptomatic salmonellosis.

Carrier state

Individuals who recover from infection can harbor the organisms in the gallbladder, which becomes the site of chronic carriage. Such individuals excrete the organisms in their feces either continuously or intermittently; nevertheless, they become an important reservoir for susceptible persons. The carrier state may be terminated by antimicrobial therapy if gallbladder infection is not evident. Otherwise, cholecystectomy has been the only solution to the chronic state of enteric carriers.

Shigella

Based on molecular analysis, the genera *Shigella* and *Escherichia* are so closely related that they should be a single genus. However, for medical purposes and because of the useful association of the genus epithet with the distinct disease shigellosis or **bacillary dysentery**, they remain as separate genera. Both genera belong to the family Enterobacteriaceae. However, *Shigella* spp. are not members of the normal GI microbiota, and all *Shigella* spp. can cause bacillary dysentery. The genus *Shigella* is named after the Japanese microbiologist Kiyoshi Shiga, who first isolated the organism in 1896. The organism, descriptively named *Shigella dysenteriae*, causes the enteric disease bacillary dysentery. The disease is characterized by the presence of blood, mucus, and pus in the stool. Characteristics of *Shigella* spp. include:

- They are nonmotile.
- Except for certain types of *S. flexneri*, they do not produce gas from glucose.
- They do not hydrolyze urea.
- They do not produce H_2S .
- They do not decarboxylate lysine.

In contrast with *Escherichia* spp., *Shigella* spp. do not use acetate or mucate as a source of carbon. Table 19.9 shows the biochemical characteristics of *Shigella* spp. *S. sonnei* is unique in its ability to decarboxylate ornithine. Most isolates also slowly ferment lactose, producing delayed-positive lactose

Table 19.9 Biochemical and serologic differentiation of *Shigella* species

Test	<i>S. dysenteriae</i>	<i>S. flexneri</i>	<i>S. boydii</i>	<i>S. sonnei</i>
Mannitol fermentation	–	+	+	+
ONPG	V	–	V	+
Ornithine decarboxylase	–	–	–	+
Serogroup	A	B	C	D

–, $\geq 9\%$ of strains positive; +, $\geq 90\%$ of strains positive; ONPG, o-Nitrophenyl- β -D-galactopyranoside; V, 10% to 89% of strains positive.

Data from Farmer, J. J., et al. (2007). Enterobacteriaceae: introduction and identification. In P. R. Murray, et al. (Eds.), *Manual of clinical microbiology* (9th ed.). Washington, DC: ASM Press.

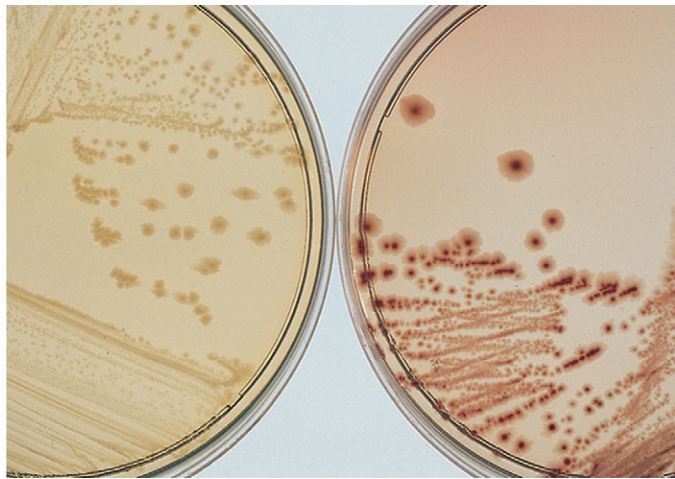


Fig. 19.10 Left, Lactose-negative appearance of *Shigella sonnei* growing on MacConkey (MAC) agar at 18 to 24 hours of incubation. Right, Lactose-positive appearance of *S. sonnei* growing on MAC agar after 48 hours of incubation.

fermentation with the formation of pink colonies on MAC agar but only after 48 hours of incubation. *S. sonnei* is positive for o-nitrophenyl- β -D-galactopyranoside (ONPG). These two key positive reactions help distinguish it from the other three species. Fig. 19.10 illustrates the growth of *S. sonnei* on MAC agar after 24 hours and 48 hours of incubation. On differential and selective media used primarily to isolate intestinal pathogens, shigellae generally appear as clear, nonlactose-fermenting colonies at 24 hours. Shigellae are fragile organisms; they are susceptible to the various effects of physical and chemical agents, such as disinfectants and high concentrations of acids and bile. Because they are susceptible to the acid pH of stool, feces suspected of containing *Shigella* organisms should be plated immediately onto laboratory media to increase recovery of the organism.

Antigenic structures

The genus consists of four biochemically similar species. *Shigella* spp. are also divided into four major O antigen serogroups (see Table 19.9). Several serotypes exist within each

species, except for *S. sonnei*, which has only one serotype. All *Shigella* spp. possess O antigens, and certain strains can possess K antigens. *Shigella* K antigens, when present, interfere with the detection of the O antigen during serologic grouping. The K antigen is heat labile and may be removed by boiling a cell suspension. The shigellae are nonmotile; therefore they lack H antigens.

Clinical infections

Shigellosis is the most important cause of bloody diarrhea worldwide. Annually, the World Health Organization estimates at least 80 million people suffer from bloody diarrhea caused by shigellosis, and it causes 700,000 deaths. Although shigellosis is endemic to many countries, about 99% of cases occur in developing countries. Most cases (about 70%) and deaths (about 60%) occur in children younger than 5 years of age. In the United States, an estimated 450,000 cases occur yearly.

Although all *Shigella* spp. can cause dysentery, species vary in epidemiology, mortality rate, and severity of disease. In the United States and Europe, *S. sonnei* is the predominant isolate followed by *S. flexneri*. In industrialized countries, including the United States, shigellosis is probably underreported because most patients are not hospitalized and usually recover from the infection without culture to identify the etiologic agent. *S. sonnei* infection is usually a short, self-limiting disease characterized by fever and watery or bloody diarrhea.

The demographics of *S. flexneri* infection have changed during recent years from a disease affecting mostly young children to one producing infections in young adults (approximately 25 years of age). This observation was made simultaneously with the recognition of gastroenteritis in men who have sex with men, in which *S. flexneri* has been the leading isolate. Persons with HIV infection are also at increased risk. Conversely, in many developing countries, *S. dysenteriae* and *S. boydii* are the most common isolates. *S. dysenteriae* type 1, a common producer of Shiga toxin, remains the most virulent species, with significant morbidity and high mortality. Reports exist of mortality rates of 5% to 10%, and perhaps even higher, resulting from *S. dysenteriae* type 1, particularly among undernourished children during epidemic outbreaks.

Humans and other large primates are the only known reservoir of *Shigella* spp. Transmission can occur by direct person-to-person contact and via the fecal-oral route, with carriers as the source. Shigellae can also be transmitted by flies, fingers, and food or water contaminated by feces from infected persons. Because of the low infective dose required to produce the disease (10 to 100 bacilli), shigellosis is highly communicable. Personal hygiene plays a major role in the transmission of *Shigella* spp., and certain groups are affected more than others. Young children in daycare centers, particularly infants younger than 1 year of age, are most susceptible. Most disturbing are the reports of multidrug-resistant *S. sonnei* outbreaks in daycare centers in several states. *Shigella* is also seen in people living in crowded and inadequate housing. Multiple *Shigella* outbreaks associated with passengers on cruise ships from various cruise lines have also been reported.

The clinical manifestations of shigellosis vary from asymptomatic to severe. The initial symptoms, marked by high fever, chills, abdominal cramps, and pain accompanied by

tenesmus, appear approximately 24 to 48 hours after ingestion of the organisms. The organisms, which originally multiply in the small intestine, move toward the colon, where they may be isolated 1 to 3 days after the infection develops. Bloody stools containing mucus and numerous leukocytes follow the watery diarrhea as the organisms invade the colonic tissues and cause an inflammatory reaction. Bacillary dysentery caused by *Shigella* spp. is marked by penetration of intestinal epithelial cells after attachment of the organisms to mucosal surfaces, local inflammation, shedding of the intestinal lining, and formation of ulcers after epithelial penetration. All *Shigella* spp. contain chromosomal genes for an enterotoxin and a cytotoxin. *S. dysenteriae* type 1 also carries the gene for Shiga toxin derived from a phage that is incorporated into the chromosome. Clinical manifestations are caused by penetration of gastric epithelial cells and production of toxins.

In dysentery caused by *S. dysenteriae* type 1, patients experience more severe symptoms. Bloody diarrhea that progresses to dysentery may appear within a few hours to a few days. Patients experience extremely painful bowel movements, which contain predominantly mucus and blood. In young children, abdominal pain is quite intense, and rectal prolapse may result from excessive straining. Severe cases of shigellosis can become life-threatening as extraintestinal complications develop. One of the most serious complications is ileus, an obstruction of the intestines, with marked abdominal dilation, possibly leading to toxic megacolon.

Although *Shigella* spp. infrequently penetrate the intestinal mucosa and disseminate to other body sites, it has been reported that 4% of severely ill hospitalized patients in Bangladesh have bacteremia caused by *S. dysenteriae* type 1. *S. flexneri* bacteremia and bacteremia resulting from other enteric organisms occur, presumably predisposed by ulcers initiated by the shigellae. Other complications of shigellosis include seizures, which may occur during any *Shigella* sp. infection. HUS is another complication among the shigellae exclusively associated with *S. dysenteriae* type 1 shigellosis, although about 80% of HUS cases in the United States are caused by EHEC. The effects of shigella toxin have been implicated as the mechanism responsible for the signs of disease, and it has been reported that the detectable toxin levels produced by *S. dysenteriae* type 1 are higher than those produced by other *Shigella* spp.

Yersinia

The genus *Yersinia*, in the family Yersiniaceae, currently consists of 25 named species; most are considered environmental isolates. Although many have been recovered from humans, only three species are considered human pathogens. *Y. pestis* is the causative agent of plague, and *Y. pseudotuberculosis* and *Y. enterocolitica* have caused sporadic cases of gastroenteritis; mesenteric lymphadenitis, especially in children; and generalized septicemic infections in immunocompromised hosts. All three species are regarded as zoonotic agents. The DNA relatedness between *Y. pestis* and *Y. pseudotuberculosis* is about 95%; however, they produce different clinical syndromes. *Y. enterocolitica* produces gastroenteritis that can mimic appendicitis. It has also been found to be the cause of diarrhea in numerous community outbreaks. The other members of the genus *Yersinia* are found in water, soil, and lower animals; isolates occasionally have been found in wounds and the urine of

humans. There is no evidence that species other than *Y. enterocolitica* cause intestinal disease. However, other *Yersinia* spp. have been isolated in up to 20% of stool samples from patients with diarrhea in the absence of other enteric pathogens.

Yersinia pestis

The causative agent of plague still exists in areas where reservoir hosts are found. The World Health Organization reported 3248 plague cases, with 584 deaths, between 2010 and 2015. In the United States, an average of 7 cases are reported annually, primarily in the southwestern region. Plague is a disease primarily of rodents. It is transmitted to humans by bites of fleas, which are its most common and effective vectors. In humans, plague can occur in three forms: the bubonic or glandular form; the septicemic form; and the pneumonic form. The bubonic form, the most common, usually results from the bite of an infected flea. Characteristic symptoms appear 2 to 6 days after infection. The symptoms include sudden onset of high fever, chills, and headache with swollen, painful regional lymph nodes known as buboes. Pneumonic plague can be a primary infection if the bacteria are inhaled, or it can be secondary to bubonic plague. This is the only form spread from person to person, and subsequent epidemic outbreaks can arise from respiratory transmission of the organisms. The septicemic form occurs when the bacteria are directly inoculated into the bloodstream without regional infection. Typical symptoms include fever, chills, shock, extreme weakness, abdominal pain, and sometimes bleeding into the skin leading to a rash. The fatality rate in pneumonic and septicemic plague is high—approaching 100%—in untreated patients.

Y. pestis is a nonmotile gram-negative, short, plump bacillus. When stained with methylene blue or Wayson stain, it shows intense staining at each end of the bacillus, referred to as *bipolar staining*, which gives it a “safety-pin” appearance. *Y. pestis* can be isolated on routine culture media. Although it grows at 37°C, its optimal growth temperature is 25° to 30°C. Further information relating its role as a potential bioterrorism agent can be found in [Chapter 30](#).

Yersinia enterocolitica

Human infections resulting from *Y. enterocolitica* have occurred worldwide, predominantly in Europe. The CDC estimates that 117,000 cases occur annually in the United States. It is the most commonly isolated species of *Yersinia*. The organism has been found in a wide variety of animals, including domestic swine, cats, and dogs. The role of pigs as a natural reservoir has been greatly emphasized in Europe. Other animal reservoirs have also been identified, and cultures from environmental reservoirs, such as water from streams, have yielded the organism.

Human infections can occur from contact with household pets; by the fecal-oral route; and after ingestion of contaminated food (often pork, vacuum-packed deli meat, beef, lamb, and chicken), unpasteurized milk, and contaminated water. There are several reports of gastroenteritis, especially in infants who were infected by caretakers who had improperly handled raw pork chitterlings (pork intestines) during food preparation procedures. A major concern regarding the potential risk of transmitting this organism is its ability to survive in cold temperatures; food refrigeration is an ineffective preventive measure. In addition, *Y. enterocolitica* sepsis

associated with the transfusion of contaminated packed red blood cells has been reported.

Y. enterocolitica infections manifest in several forms: acute gastroenteritis, appendicitis-like syndrome (mesenteric lymphadenitis), terminal ileitis, and less frequently, septicemia, arthritis, and erythema nodosum. The incidence of systemic infection is higher among children, older adults, or those with underlying diseases, such as liver cirrhosis, diabetes, acquired immunodeficiency syndrome, leukemia, aplastic anemia, and other hematologic conditions. Cases of liver abscess and acute infective endocarditis caused by *Y. enterocolitica* have also been reported. Acute enteritis, the most common form of the infection, is characterized by acute gastroenteritis with fever accompanied by headache, abdominal pain, nausea, and diarrhea. Stools may contain blood. This form of infection, which often affects infants and young children between 1 and 5 years of age, is usually mild and self-limiting in about 7 days.

The clinical form that mimics acute appendicitis occurs primarily in older children and adults. Patients present with severe abdominal pain and fever; the abdominal pain is concentrated in the right lower quadrant. Enlarged mesenteric lymph nodes and an inflamed ileum and appendix are common findings in cases of *Y. enterocolitica* infections. Arthritis is an uncommon extraintestinal form of *Y. enterocolitica* infection, usually after a GI episode or an appendicitis-like syndrome. This form of yersiniosis has been reported more often in adults than in children. Erythema nodosum is an inflammatory reaction caused by *Y. enterocolitica* characterized by tender, red nodules that may be accompanied by itching and burning. The areas involved include the anterior portion of the legs; some patients have reported nodules on their arms. The syndrome is reported to be more common in female patients compared with male patients.

Y. enterocolitica morphologically resembles other *Yersinia* spp., appearing as gram-negative coccobacilli with bipolar staining. The organism also grows on routine isolation media, such as SBA and MAC agar. It has an optimal growth temperature of 25° to 30° C. *Y. enterocolitica* is clearly motile at 25° C but not at 35° C. Appropriate cultures on a specific *Yersinia* medium at 25° C should be performed in diarrheal outbreaks of unknown etiology. Cold enrichment can be used to increase the recovery in fecal samples suspected of containing this organism. Fecal material is inoculated into isotonic saline and kept at 4° C for 1 to 3 weeks, with weekly subculturing to selective agar for *Yersinia*.

Cefsulodin-irgasan-novobiocin (CIN) agar, a selective medium to detect the presence of *Y. enterocolitica*, incorporates cefsulodin, irgasan, novobiocin, bile salts, and crystal violet as inhibitory agents. This medium, which inhibits normal intestinal microbiota more than MAC agar, provides better opportunities to recover *Y. enterocolitica* from feces. Different formulations of CIN are available; for example, *Yersinia*-selective agar (YSA) base (BD Diagnostic Systems, Sparks, MD) has a differential property (mannitol) added to the medium. Fermentation of mannitol results in a decrease in pH around the colony, causing the pH indicator, neutral red, to turn red at the center of the colony and the bile to precipitate. Nonfermentation of mannitol produces a colorless, translucent colony. *Y. enterocolitica* ferments mannitol. A modified formulation of the original CIN medium, known as CIN II, or BD *Aeromonas Yersinia* Agar (Becton-Dickinson,

Franklin Lakes, NJ), can be used to simultaneously isolate most *Aeromonas* spp. from stool samples.

Yersinia pseudotuberculosis

Y. pseudotuberculosis, like *Y. pestis*, primarily is a pathogen of rodents, particularly guinea pigs. In addition to farm and domestic animals, birds are natural reservoirs; turkeys, geese, pigeons, doves, and canaries have yielded positive cultures for this organism. The bacteria are also found in the environment, primarily in colder climates, like northern Europe as well as Asia, including Japan. *Y. pseudotuberculosis* causes a disease characterized by caseous swellings called *pseudotubercles*. The disease is often fatal in animals.

Human infections, which are rare, are associated with close contact with infected animals, or their feces, or ingestion of contaminated drink and foodstuff. When the organisms are ingested, they spread to the mesenteric lymph nodes, producing abdominal pain and mesenteric lymphadenitis that is usually self-limiting. The clinical manifestations can include septicemia, primarily in immunocompromised individuals. Infections are most common in children and young adults. *Y. pseudotuberculosis* appears as a typical-looking plague bacillus. It can be differentiated from *Y. pestis* by its motility at 18°

Table 19.10 Differentiation of selected species within the genus *Yersinia*

Test	<i>Y. pestis</i>	<i>Y. enterocolitica</i>	<i>Y. pseudotuberculosis</i>
Indole	–	d	–
Methyl red	+	+	+
Voges-Proskauer			
25°C	–	d	–
37°C	–	–	–
Motility			
25°C	–	+	+
37°C	–	–	–
β-Galactosidase	+	+	+
Christensen urea	–	+	+
Phenylalanine deaminase	–	–	–
Ornithine decarboxylase	–	+	–
Acid produced from			
Sucrose	–	+	–
Lactose	–	–	–
Rhamnose	–	– or + ^a	+
Melibiose	–	– or + ^a	+
Trehalose	–	+	+
Cellobiose	–	+	–

+, ≥90% positive reaction within 1 or 2 days; –, no reaction (≥90%) in 30 days; – or +, most strains negative, some cultures positive; + or (+), most reactions occur within 1 or 2 days, some are delayed; + or –, most cultures positive, some strains negative; d, different reactions.

^aTest results at 25° C.

Modified from Washington, J. (1981). *Laboratory procedures in clinical microbiology* (2nd ed.). New York: Springer-Verlag.

to 22° C, production of urease, and ability to ferment rhamnose. Table 19.10 shows differentiating characteristics among *Yersinia* spp.

Other genera of the order Enterobacterales

Budvicia

Based on DNA hybridization, *Budvicia* is a group of related organisms in the family Budviciaceae; however, they are not as closely related to the other members of Enterobacterales. These organisms are usually found in water; however, *Budvicia aquatica* has been isolated in clinical specimens.

Buttiauxella

The genus *Buttiauxella*, in the family Enterobacteriaceae, consists of eight species isolated from water. Only *B. agrestis* and *B. noackiae* have been isolated from human specimens. Biochemically, these organisms resemble *Citrobacter* and *Kluyvera* species, but DNA hybridization distinctly differentiates *Buttiauxella* from both genera.

Cedecea

The genus *Cedecea*, in the family Enterobacteriaceae, is composed of four named species: *C. colo*, *C. davisae*, *C. lapagei*, and *C. neteri*. Most have been recovered from sputum, blood, urine, and wounds. Of the four, *C. davisae* is the most common clinical isolate.

Ewingella

Within the family Yersiniaceae, *Ewingella americana* is the only named species in the genus *Ewingella*. Most isolates have come from human blood cultures or respiratory specimens and exhibit resistance to multiple antimicrobial agents.

Leclercia

The name *Leclercia* was proposed in 1986 for 58 isolates from human clinical specimens including blood, urine, sputum, and feces and 27 isolates from nonhuman sources. It has been isolated more recently in pure culture from septicemia and wounds. The single species is *L. adecarboxylata*, which can have a yellow pigment but only on initial isolation. Although it has similar IMViC reactions to *E. coli*, it is negative for lysine and ornithine decarboxylase and arginine dihydrolase (i.e., triple decarboxylase–negative). It resides in the family Enterobacteriaceae.

Leminorella

Leminorella was proposed as a genus, in the family Budviciaceae, with two species: *L. grimontii* and *L. richardii*. These organisms produce H₂S and have shown weak reactions with *Salmonella* antisera. However, biochemical testing differentiates *Leminorella* from *Salmonella*; *Leminorella* spp. are

relatively inactive. The clinical significance of these organisms is unknown; however, they have been isolated from patients with hospital-acquired infections.

Moellerella

The genus *Moellerella*, within the family Morganellaceae, contains one species, *M. wisconsensis*. The species is positive for citrate, methyl red, lactose, and sucrose. It is negative for lysine, ornithine, arginine decarboxylase, and indole, and it resembles *E. coli* growing on enteric media. The clinical significance of this organism has not been established, although it has been isolated from feces in cases of diarrhea, infected gallbladders, and a bronchial aspirate.

Rahnella

Rahnella, in the family Yersiniaceae, is the name given to a group of seven named species that are psychrotolerant, growing at 4°C. These organisms have no single characteristic that distinguishes them from the other members of the Enterobacterales. They resemble *P. agglomerans*; however, they can be distinguished by their weak phenylalanine deaminase reaction; negative result for potassium cyanide (KCN), gelatin, lysine, ornithine, and motility; and lack of yellow pigmentation. They have been occasionally isolated from human clinical specimens, including wound infections, bacteremias, feces from patients with acute gastroenteritis, and septicemia, especially from immunocompromised patients.

Tatumella

The genus *Tatumella*, in the family Erwiniaceae, contains six species. These organisms are unusual for the Enterobacterales in several ways: stock cultures can be kept frozen in sheep red blood cells or freeze-dried, but they die in a few weeks on agar slants; they show more biochemical reactions at 25° C than at 35° C; they are motile at 25° C but not at 35° C; and they demonstrate large 15- to 36-mm zones of inhibition around penicillin disks. In addition, *Tatumella* isolates are slow growing, produce tiny colonies, and are relatively nonreactive in laboratory media. These organisms have been isolated from human sources, especially sputum, and may be a rare cause of infection. Their reservoir is unknown.

Laboratory diagnosis of the Enterobacterales

Specimen collection and transport

Members of the order Enterobacterales can be isolated from a wide variety of sterile and nonsterile clinical samples. Often these bacterial species are isolated with other organisms, including more fastidious pathogens. To ensure isolation of both opportunistic and fastidious pathogens, laboratories must provide appropriate transport media, such as Cary-Blair, Amies, or Stuart media. Microbiology personnel must encourage immediate transport of clinical samples to the laboratory for processing, regardless of the source of the clinical specimen.

Isolation and identification

To determine the clinical significance of the isolate, the microbiologist must consider the site of origin. Generally, enteric opportunistic organisms isolated from sites that are normally sterile are highly significant. However, careful examination is critical of organisms recovered from, for example, the respiratory tract, urogenital tract, stool, and wounds in open sites that are inhabited by other endogenous microbiota. Members of the order Enterobacterales are routinely isolated from stool cultures; complete identification should be directed only toward true intestinal pathogens. Sputum cultures from hospitalized patients can contain enteric organisms that may require complete identification. Multiplex PCR assays can detect multiple pathogens in clinical specimens. For example, the FilmArray assay can detect 22 common GI pathogens, including bacteria, parasites, and viruses, in about 1 hour. Another FilmArray assay detects 30 microorganisms, including several members of the order Enterobacterales, and 10 drug-resistance genes in a positive blood culture.

Direct microscopic examination

In contrast with gram-positive bacteria, in which microscopic morphology may help provide a presumptive identification, the microscopic characteristics of enterics are indistinguishable from other gram-negative bacilli. However, smears prepared directly from sterile sites, like CSF and other body fluids or exudates, can be examined microscopically for the presence of gram-negative bacteria. Although this examination is non-specific for enteric organisms, this presumptive result can aid the clinician in the preliminary diagnosis of the infection, and appropriate empiric therapy can be instituted immediately.

Direct smears prepared from samples containing endogenous microbiota, such as sputum, do not provide valuable information because their significance cannot be fully assessed unless the gram-negative bacteria are prevalent and endogenous inhabitants are absent. Direct smear examination of stool samples is not helpful in identifying enteric pathogens but may reveal the presence of inflammatory cells. This information may be helpful in determining whether a GI disease is a toxin-mediated or invasive process.

Culture

Members of the Enterobacterales are facultative anaerobes, and most clinically significant species grow at an optimal temperature of 35° to 37° C. Certain species can grow at low temperatures (1° to 5° C, such as *Serratia* and *Yersinia*) or tolerate high temperatures (45° to 50° C, such as *E. coli*). Colonies become visible on nonselective and differential media after 18 to 24 hours of incubation. Most laboratories use a wide variety of nonselective media, such as SBA and CHOC agar, as well as selective media, such as MAC agar, to recover enteric organisms from wounds, respiratory tract secretions, urine, and sterile body fluids. On CHOC agar or SBA plates, enteric bacteria produce large, grayish, smooth colonies. On SBA, colonies may be β -hemolytic or nonhemolytic.

Screening stool cultures for pathogens

Because of the mixed microbial biota of fecal specimens, efficient screening methods must be used for the recovery

and identification of enteric pathogens. All fecal specimens should be routinely screened for *Salmonella*, *Shigella*, STEC, and *Campylobacter* (see Chapter 20). Laboratory protocols and media should also be available for *Yersinia*, *Aeromonas*, *Vibrio*, and *P. shigelloides* when appropriate or requested. Screening routinely for the additional organisms may not be cost-effective, but they should be addressed based on patient history (e.g., travel near coastal areas or developing countries where certain organisms are endemic) and gross description of the specimen (bloody or watery).

Stool specimens contain enteric organisms as normal intestinal microbiota; in processing stool samples, laboratories develop their own protocol for the maximum recovery of enteric pathogens. Fecal pathogens are generally nonlactose fermenters (NLFs). These organisms appear as clear or colorless and translucent colonies on MAC agar. However, many of the bacteria that compose common fecal microbiota also appear as NLFs (e.g., *Proteus* and *Providencia*) as well as delayed lactose-fermenting organisms (e.g., *Serratia* and *Citrobacter*). For this reason, it is necessary to use screening tests to differentiate these organisms from pathogens found in feces. One approach is to take a well-isolated, NLF colony and perform a screening battery of tests consisting first of an oxidase test and the inoculation of lysine iron agar (LIA) and TSI agar slants (Table 19.11). If the screening tests rule out pathogenic organisms, the process is complete, and the culture is discarded (after 48 hours of incubation).

Most clinical microbiologists inoculate stool samples on highly selective media, such as HE or XLD agars, in addition to MAC and SMAC agars for *E. coli* O157:H7. An enrichment broth (e.g., selenite, GN) has been traditionally inoculated to enhance recovery. As previously mentioned, CIN II agar can serve the dual purpose of screening for *Y. enterocolitica*, and most *Aeromonas* spp. *Y. enterocolitica* produces pale rose colonies with a dark red center ("bull's eye"), whereas *Aeromonas* spp. form colorless to rose-colored colonies with a rose center. One must remember that *Yersinia* is oxidase-negative, *Aeromonas* is oxidase-positive, and testing for this trait must be done on SBA, not on selective or differential media. The oxidase-positive *P. shigelloides*, a potential enteric pathogen, produces opaque colonies without a pink center (mannitol nonfermenter) on CIN agar.

On HE agar, lactose-fermenting species produce yellow colonies, whereas NLFs such as *Shigella* spp. produce green colonies (Fig. 19.11). *Salmonella* spp. are NLFs, creating green colonies with black centers from H₂S production. However, *Proteus* spp. are NLFs, and species that produce H₂S also appear green with black centers on HE agar. *Salmonella* Paratyphi is the only serotype that is H₂S negative. *C. freundii* usually produces yellow colonies with black centers. NLF species such as *Salmonella enterica*, which produces lysine decarboxylase, produce red colonies with black centers on XLD medium (Fig. 19.12).

Another selective and differential medium is *Salmonella-Shigella* (SS) agar, a light straw-colored medium in which *Salmonella* colonies appear colorless with dark black centers from H₂S production, and *Shigella* colonies appear as colorless colonies only. *E. tarda*, a potential enteric pathogen, produces colonies that resemble *Salmonella*: NLF and H₂S producer. On CHROMagar *Salmonella*, *Salmonella* isolates produce mauve-colored colonies (Fig. 19.13) resulting from the activity of an esterase on a patented substrate. Other members of

Table 19.11 Stool culture screening for enteric pathogens using triple sugar iron and lysine-iron agar in combination

LIA reactions	TSI Reactions							
	K/A H ₂ S	K/AG H ₂ S	K/AG	K/A	A/A H ₂ S	A/AG	A/A	K/K
R/A		<i>P. vulgaris</i>	<i>M. morganii</i>	<i>M. morganii</i>	<i>P. vulgaris</i>	—	<i>Providencia</i>	—
		<i>P. mirabilis</i>	<i>Providencia</i>	<i>Providencia</i>	<i>P. mirabilis</i>			
K/K H ₂ S	<i>Salmonella</i> ^a	<i>Salmonella</i> ^a	<i>Salmonella</i> ^a	<i>Salmonella</i> ^a	—	—	—	—
	<i>Edwardsiella</i>	<i>Edwardsiella</i> ^a						
K/K	<i>Salmonella</i>	—	<i>Hafnia</i>	<i>Salmonella</i> ^a	—	<i>Klebsiella</i>	<i>Serratia</i>	<i>Pseudomonas</i> ^b
			<i>Klebsiella</i>	<i>Plesiomonas</i> ^b		<i>Enterobacter</i>		
			<i>Serratia</i>	<i>Hafnia</i>		<i>E. coli</i>		
K/A H ₂ S	—	<i>Salmonella</i> ^a	—	<i>Serratia</i>	—	—	—	—
K/A	—	<i>Citrobacter</i>	<i>Salmonella</i> ^a	<i>Shigella</i> ^a	<i>Citrobacter</i>	<i>Aeromonas</i> ^{a,b}	<i>Aeromonas</i> ^{a,b}	—
			<i>Shigella</i>	<i>Yersinia</i>		<i>E. coli</i>	<i>Yersinia</i>	
			<i>Aeromonas</i> ^b	<i>Aeromonas</i> ^b		<i>Citrobacter</i>	<i>Citrobacter</i>	
			<i>E. coli</i>	<i>E. coli</i>		<i>Enterobacter</i>	<i>Enterobacter</i>	
			<i>Enterobacter</i>	<i>Enterobacter</i>				
			<i>Citrobacter</i>					

^aResults of TSI and LIA reactions in this category indicate a potential pathogen; additional tests must be performed.

^bOxidase positive.

A, Acid; G, gas; H₂S, hydrogen sulfide; K, alkaline; LIA, lysine-iron agar; R, deamination (red slant); TSI, triple sugar iron.

Data from Microbiology Laboratory, The Ohio State University Hospitals and Maureta Ott, Columbus, OH.



Fig. 19.11 Clear, green colonies of *Shigella* growing on Hektoen enteric agar. (Courtesy R. Abe Baalness.)

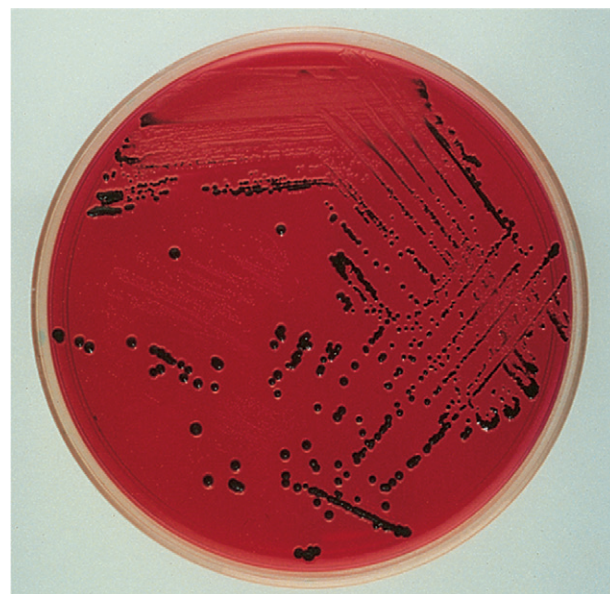


Fig. 19.12 H₂S-producing colonies of salmonellae growing on xylose-lysine-desoxycholate agar. (Courtesy American Society for Clinical Laboratory Science, Education and Research Fund, Inc., 1982.)

the order Enterobacterales produce blue or white colonies. Each laboratory must decide which media work best in their situation and patient population. Many larger clinical laboratories employ multiplex PCR (e.g., FilmArray) directly on fecal samples, thus eliminating the need for stool cultures.

Identification

The identification of members of the order Enterobacterales can be accomplished in several ways. The clinical

microbiologist can presumptively determine utilization of carbohydrates by observing the colony morphology of the isolate on differential or selective media such as MAC, SMAC, CIN, HE, or XLD. To identify an isolate, the clinical microbiologist first must determine whether the isolate belongs to the order Enterobacterales. All members of the order (1) are oxidase negative except *P. shigelloides*, (2) ferment glucose, and (3) reduce nitrate to nitrite except *Photorhabdus* (rare human isolate) and *Xenorhabdus*

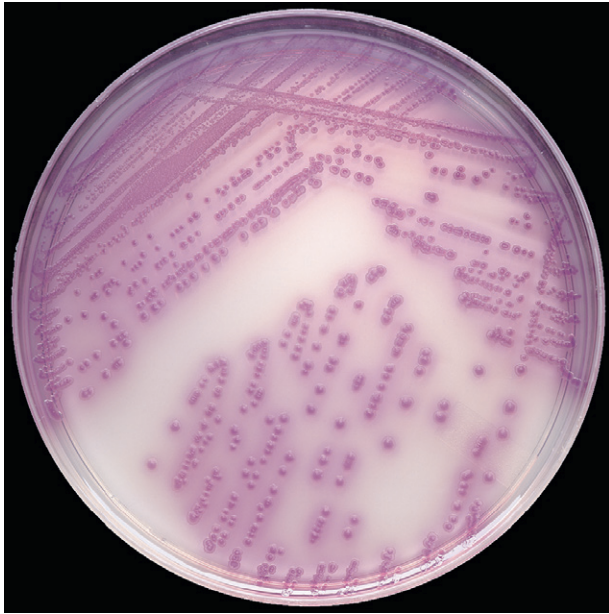


Fig. 19.13 *Salmonella* growing on CHROMagar *Salmonella* differential agar. (Courtesy BD Diagnostic Systems, Sparks, MD.)

(environmental isolate). Gram-negative isolates, especially NLFs, should be tested for cytochrome oxidase production. The oxidase test should always be performed using young growth from an SBA plate. Testing colonies from highly selective media such as CIN can give a false-negative reaction, whereas MAC and EMB agars may give the appearance of a false-positive reaction from the pH indicators and dyes present in the media.

A few laboratories choose to use conventional biochemical tests in tubes, whereas others may prefer miniaturized or automated commercial identification systems. See [Chapter 9](#) for a thorough description of the currently available rapid and automated commercial identification systems. The use of conventional biochemical tests in tubes is cumbersome because of the large number of biochemical tests available.

Sometimes, a few biochemical tests, such as LIA and TSI mentioned earlier, can be performed to rule out potential enteric pathogens. The Clinical and Laboratory Standards Institute has created several abbreviated test protocols for the identification of common bacterial isolates. For example, a gram-negative bacillus that forms nonswarming, β -hemolytic colonies and is spot indole positive is identified as *E. coli*. Occasional isolates that are not β -hemolytic must be indole-positive and lactose-positive on MAC or EMB, and they must exhibit a negative pyrrolidonyl arylamidase (PYR) test to be identified as *E. coli*.

Many clinical laboratories develop identification tables and protocols using a limited number of tests that suit their workflow and capabilities. Identification tables are based on the key features necessary to identify each genus and clinically relevant species. [Fig. 19.14](#) shows an example of a schematic diagram for the identification of commonly isolated enterics using conventional biochemical tests. [Table 19.12](#) shows the differentiating characteristics of the species, subspecies, biogroups, and serotypes of the Enterobacterales. As their costs

have decreased, the use of molecular biology assays is becoming more frequent. These assays include DNA probes, NAATs, the Microbial Identification System (MIDI, Newark, DE), and MALDI-TOF mass spectrometry.

Serologic grouping

When an isolate is biochemically identified as *Salmonella* or *Shigella*, serologic grouping of the isolate for the O serogroups (oligosaccharide somatic antigen) must be performed for confirmation. Serologic grouping is also important in the identification of enterovirulent *E. coli* strains.

Salmonella

Salmonella isolates can be typed using O, H, and Vi antigens. Based on O antigens, *Salmonella* can be placed into major serogroups designated by capital letters. Approximately 47 somatic O antigenic groups exist; however, 95% of *Salmonella* isolates from humans belong to serogroups A through E1. Originally, serogroups were designated by letters of the alphabet. However, because there are more serogroups than letters, numbers were also used. Currently, it is recommended to simply use the O antigen number designation for the serogroup and not use letters. For example, all members of serogroup A express O antigen 2. The current designation for the serogroup is O:2 ([Table 19.13](#)).

It is imperative for laboratories to be able to identify *Salmonella* serotype Typhi, in particular, serologically and other members of *Salmonella* in serogroups A to G (O:13). Other isolates can be reported as “biochemically compatible with *Salmonella*” and submitted to a reference laboratory for further identification. If an isolate is suspected of being *Salmonella* Typhi, *Salmonella* Paratyphi, or *Salmonella* Choleraesuis, it must be biochemically identified and serologically confirmed because of the medical implications. Identification is very important in providing proper therapy for the patient and in limiting possible complications.

To perform serologic grouping by the slide technique, a suspension from a pure culture of the organism is prepared in sterile saline solution. Serologic typing is best performed on a colony taken from a pure culture growing on nonselective media, such as an SBA plate, although TSI or MAC agar can be used for presumptive serologic identification.

A slide with wells is easy to use for the agglutination test. A regular microscope slide may be used by marking separate circles with a wax pencil. The laboratory scientist places one drop of antisera on the appropriately labeled slide. One drop of bacterial suspension is added to each drop of antisera for a direct agglutination assay. Antisera kits usually consist of a polyvalent A to G, Vi, and individual antisera for serogroups A, B, C1–C4, C2–C3, D, E, and G. Latex agglutination kits offer improved visibility of agglutination reactions; this is an example of reverse passive agglutination.

The Vi antigen is found on *Salmonella* Typhi and occasionally other serotypes and can mask the O antigens. In the event of a positive agglutination in the Vi antisera with no agglutination in the other groups, the suspension should be heated to 100° C for 10 minutes to inactivate the capsular Vi antigen. The suspension is then cooled and retested with antisera A to G. Larger laboratories usually maintain antisera to serogroup

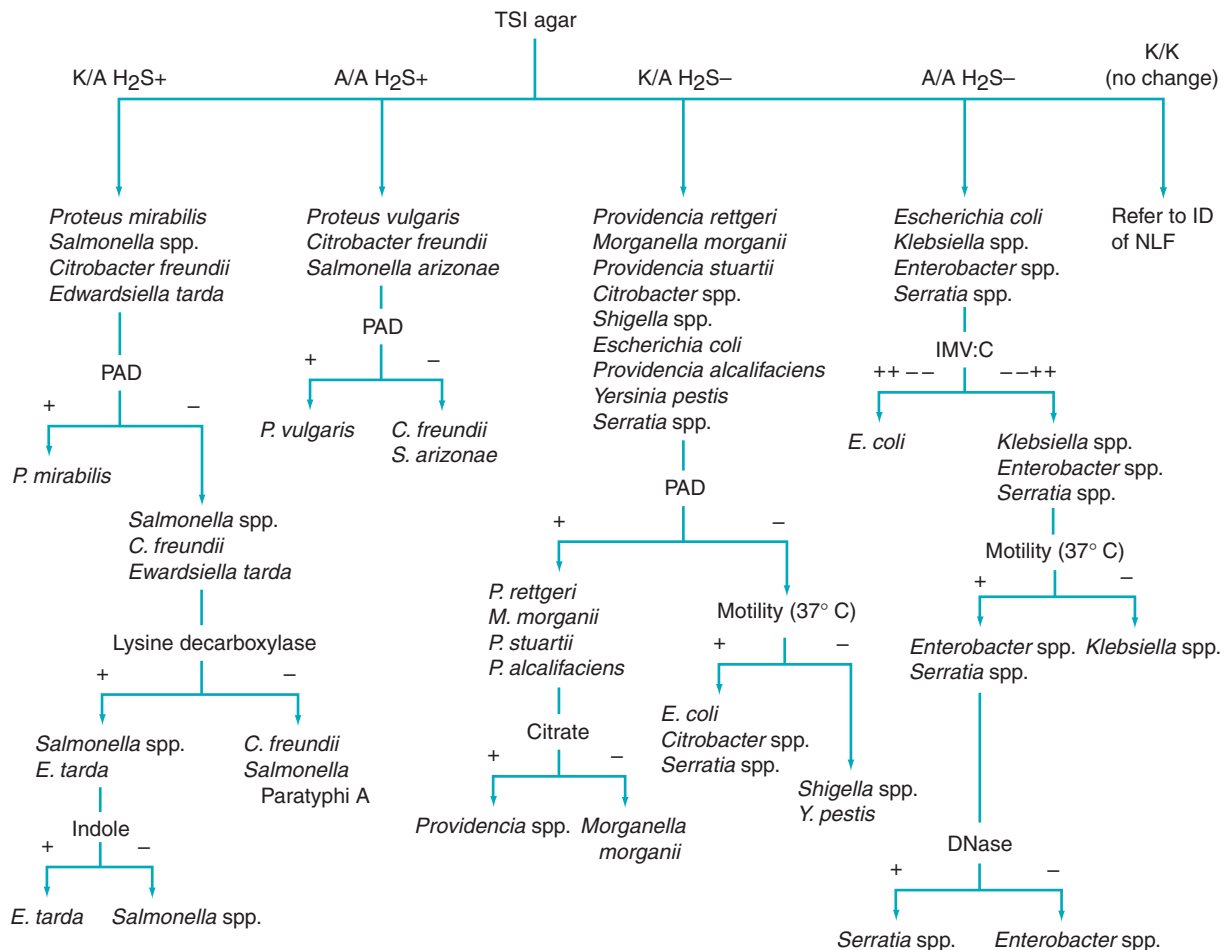


Fig. 19.14 Flowchart for the presumptive identification of commonly encountered Enterobacterales on triple sugar iron (TSI) agar. A, Acid; IMV:C, indole, methyl red, Voges-Proskauer, citrate; K, alkaline; NLF, nonlactose fermenter; PAD, phenylalanine deaminase. (Data from Koneman, E., et al. [1997]. *Color atlas in diagnostic microbiology* [5th ed.]. Philadelphia: Lippincott-Raven.)

salmonellae for all somatic types. H antigen (flagellar) typing and next-generation sequencing technology are usually performed in a state or reference laboratory providing epidemiologic information in outbreaks.

Shigella

Similar serogrouping procedures may be used in the serologic testing for *Shigella*. Serologic grouping of *Shigella* is also based on the somatic O antigen. However, *Shigella* spp. are nonmotile and do not express H antigen. Antigenic typing is necessary to differentiate the *Shigella* spp. from inactive *E. coli* and to confirm the identification of individual species. *Shigella* spp. belong to one of four serogroups: A (*S. dysenteriae*), B (*S. flexneri*), C (*S. boydii*), and D (*S. sonnei*). The O antisera used for serogrouping are polyvalent, containing several serotypes within each group (except for group D, which contains only a single serotype). If agglutination fails, the suspension must be heated to remove the capsular antigen that might be

present, and subsequently the agglutination test procedure is repeated.

Plesiomonas shigelloides

P. shigelloides can be presumptively differentiated from similar genera with several key tests. The positive oxidase activity separates it from other Enterobacterales, sensitivity to the agent O/129 separates it from *Aeromonas*, and its ability to ferment inositol separates it from all *Aeromonas* and almost all *Vibrio* spp. It can also be separated from the halophilic *Vibrio* spp. by its ability to grow in nutrient broth with 0% NaCl and its inability to grow in nutrient broth with 6% NaCl.

Most biochemical identification systems include *P. shigelloides* in their databases and appear to be able to identify it with a high degree of accuracy. This is in large part because of its unique, positive trio profile of ornithine and lysine decarboxylases and arginine dihydrolase reactions, combined with the fermentation of inositol. It is also included in the PCR-based FilmArray GI panel.

Table 19.12 Biochemical reactions of selected named species of the order Enterobacterales^a

Organism	Indole production	Methyl red	Voges-proskauer	Citrate (Simmons)	H ₂ S (TSI)	Urea hydrolysis	Phenylalanine deaminase	Lysine decarboxylase	Arginine dihydrolase	Ornithine decarboxylase	Motility	Gelatin hydrolysis (22° C)	Growth in KCN	Malonate utilization	d-Glucose, acid	d-Glucose, gas	Lactose fermentation	Sucrose fermentation	d-Mannitol fermentation	Dulcitol fermentation	Salicin fermentation
<i>Budvicia aquatic</i>	0	93	0	0	80	33	0	0	0	0	27	0	0	0	100	53	87	0	60	0	0
<i>Buttiauxella agrestis</i>	0	100	0	100	0	0	0	0	0	100	100	0	80	60	100	100	100	0	100	0	100
<i>Buttiauxella noackiae</i>	33	100	0	33	0	0	100	0	67	0	100	0	100	100	100	100	0	0	100	0	100
<i>Cedecea davisae</i>	0	100	50	95	0	0	0	0	50	95	95	0	86	91	100	70	19	100	100	0	99
<i>Cedecea lapagei</i>	0	40	80	99	0	0	0	0	80	0	80	0	100	99	100	100	60	0	100	0	100
<i>Cedecea neteri</i>	0	100	50	100	0	0	0	0	100	0	100	0	65	100	100	100	35	100	100	0	100
<i>Citrobacter amalonaticus</i>	100	100	0	95	5	85	0	0	85	95	95	0	99	1	100	97	35	9	100	1	30
<i>Citrobacter braakii</i>	33	100	0	87	60	47	0	0	67	93	87	0	100	0	100	93	80	7	100	33	0
<i>Citrobacter farmeri</i>	100	100	0	10	0	59	0	0	85	100	97	0	93	0	100	96	15	100	100	2	9
<i>Citrobacter freundii</i>	33	100	0	78	78	44	0	0	67	0	89	0	89	11	100	89	78	89	100	11	0
<i>Citrobacter koseri</i>	99	100	0	99	0	75	0	0	80	99	95	0	0	95	100	98	50	40	99	40	15
<i>Citrobacter youngae</i>	15	100	0	75	65	80	0	0	50	5	95	0	95	5	100	75	25	20	100	85	10
<i>Cronobacter sakazakii</i>	11	5	100	99	0	1	50	0	99	91	96	0	99	18	100	98	99	100	100	5	99
<i>Edwardsiella hoshinae</i>	50	100	0	0	0	0	0	100	0	95	100	0	0	100	100	35	0	100	100	0	50
<i>Edwardsiella tarda</i>	99	100	0	1	100	0	0	100	0	100	98	0	0	0	100	100	0	0	0	0	0
<i>Enterobacter asburiae</i>	0	100	2	100	0	60	0	0	21	95	0	0	97	3	100	95	75	100	100	0	100
<i>Enterobacter cancerogenus</i>	0	5	100	100	0	1	0	0	94	99	99	0	98	100	100	100	10	0	100	0	92
<i>Enterobacter cloacae</i>	0	5	100	100	0	65	0	0	97	96	95	0	98	75	100	100	93	97	100	15	75
<i>Enterobacter hormaechei</i>	0	57	100	96	0	87	4	0	78	91	52	0	100	100	100	83	9	100	100	87	44
<i>Escherichia albertii</i>	0		0	0	0	0	0	100	0	100	0	0	0	0	100	100	0	0	100	0	0
<i>Escherichia coli</i>	98	99	0	1	1	1	0	90	17	65	95	0	3	0	100	95	95	50	98	60	40
<i>Escherichia fergusonii</i>	98	100	0	17	0	0	0	95	5	100	93	0	0	35	100	95	0	0	98	60	65
<i>Ewingella americana</i>	0	84	95	95	0	0	0	0	0	0	60	0	5	0	100	0	70	0	100	0	80
<i>Hafnia alvei</i>	0	40	85	10	0	4	0	100	6	98	85	0	95	50	100	98	5	10	99	0	13
<i>Klebsiella aerogenes</i>	0	5	98	95	0	2	0	98	0	98	97	0	98	95	100	100	95	100	100	5	100
<i>Klebsiella oxytoca</i>	99	20	95	95	0	90	1	99	0	0	0	0	97	98	100	97	100	100	99	55	100

Adonitol fermentation	myo-Inositol fermentation	d-Sorbitol fermentation	L-Arabinose fermentation	Raffinose fermentation	L-Rhamnose fermentation	Maltose fermentation	d-Xylose fermentation	Trehalose fermentation	Cellobiose fermentation	α -Methyl-d-glucoside fermentation	Erythritol fermentation	Esculin hydrolysis	Melibiose fermentation	d-Arabitol fermentation	Glycerol fermentation	Mucate fermentation	Tartrate, jordan's	Acetate utilization	Lipase (Corn oil)	DNase (25° C)	Nitrate nitrite	Oxidase, kovacs'	ONPG test	Yellow pigment	d-Mannose fermentation
0	0	0	80	0	100	0	93	0	0	0	0	0	0	27	0	20	27	0	0	0	100	0	93	0	0
0	0	0	100	100	100	100	100	100	100	0	0	100	100	0	60	100	60	0	0	0	100	0	100	0	100
0	0	0	100	0	100	100	100	100	100	33	0	100	0	0	0	100	100	0	0	0	100	0	100	0	100
0	0	0	0	10	0	100	100	100	100	5	0	45	0	100	0	0	0	0	91	0	100	0	90	0	100
0	0	0	0	0	0	100	0	100	100	0	0	100	0	100	0	0	0	60	100	0	100	0	99	0	100
0	0	100	0	0	0	100	100	100	100	0	0	100	0	100	0	0	0	0	100	0	100	0	100	0	100
0	0	99	99	5	100	99	99	100	100	2	0	5	0	0	60	96	96	86	0	0	99	0	97	0	100
0	0	100	100	7	100	100	100	100	73	33	0	0	80	0	87	100	93	53	0	0	100	0	80	0	100
0	0	98	100	100	100	100	100	100	100	75	0	0	100	0	65	100	93	80	0	0	100	0	100	0	100
0	0	100	100	44	100	100	89	100	44	11	0	0	100	0	100	100	100	44	0	0	100	0	89	0	100
99	0	99	99	0	99	100	100	100	99	40	0	1	0	98	99	95	90	75	0	0	100	0	99	0	100
0	5	100	100	10	100	95	100	100	45	0	0	5	10	5	90	100	100	65	0	0	85	0	90	0	100
0	75	0	100	99	100	100	100	100	100	96	0	100	100	0	15	1	1	96	0	0	99	0	100	98	100
0	0	0	13	0	0	100	0	100	0	0	0	0	0	0	65	0	0	0	0	0	100	0	0	0	100
0	0	0	9	0	0	100	0	0	0	0	0	0	0	0	30	0	25	0	0	0	100	0	0	0	100
0	0	100	100	70	5	100	97	100	100	95	0	95	0	0	11	21	30	87	0	0	100	0	100	0	100
0	0	1	100	0	100	99	100	100	100	1	0	90	0	0	1	75	0	35	0	0	100	0	100	0	100
25	15	95	100	97	92	100	99	100	99	85	0	30	90	15	40	75	30	75	0	0	99	0	99	0	100
0	0	0	100	0	100	100	96	100	100	83	0	0	0	0	4	96	13	74	0	0	100	0	95	0	100
0	0	0	100	0	0	60	0	60	0	0	0	20	0	0	100	0		100	0	0	100	0	100	0	100
5	1	94	99	50	80	95	95	98	2	0	0	35	75	5	75	95	95	90	0	0	100	0	95	0	98
98	0	0	98	0	92	96	96	96	96	0	0	46	0	100	20	0	96	96	0	0	100	0	83	0	100
0	0	0	0	0	23	16	13	99	10	0	0	50	0	99	24	0	35	10	0	0	97	0	85	0	99
0	0	0	95	2	97	100	98	95	15	0	0	7	0	0	95	0	70	15	0	0	100	0	90	0	100
98	95	100	100	96	99	99	100	100	100	95	0	98	99	100	98	90	95	50	0	0	100	0	100	0	95
99	98	99	98	100	100	100	100	100	100	98	2	100	99	98	99	93	98	90	0	0	100	0	100	1	100

Continued

Table 19.12 Biochemical reactions of selected named species of the order Enterobacterales^a—cont'd

Organism	Indole production	Methyl red	Voges-proskauer	Citrate (Simmons)	H ₂ S (TSI)	Urea hydrolysis	Phenylalanine deaminase	Lysine decarboxylase	Arginine dihydrolase	Ornithine decarboxylase	Motility	Gelatin hydrolysis (22° C)	Growth in KCN	Malonate utilization	d-Glucose, acid	d-Glucose, gas	Lactose fermentation	Sucrose fermentation	d-Mannitol fermentation	Dulcitol fermentation	Salicin fermentation
<i>Klebsiella pneumoniae</i> subsp. <i>ozaenae</i>	0	98	0	30	0	10	0	40	6	3	0	0	88	3	100	50	30	20	100	2	97
<i>Klebsiella pneumoniae</i> subsp. <i>pneumoniae</i>	0	10	98	98	0	95	0	98	0	0	0	0	98	93	100	97	98	99	99	30	99
<i>Klebsiella pneumoniae</i> subsp. <i>rhinoscleromatis</i>	0	100	0	0	0	0	0	0	0	0	0	0	80	95	100	0	0	75	100	0	98
<i>Kluyvera ascorbata</i>	92	100	0	96	0	0	0	97	0	100	98	0	92	96	100	93	98	98	100	25	100
<i>Kluyvera cryocrescens</i>	90	100	0	80	0	0	0	23	0	100	90	0	86	86	100	95	95	81	95	0	100
<i>Kluyvera georgiana</i>	100	100	0	100	0	0	0	100	0	100	100	0	83	50	100	17	83	100	100	33	100
<i>Kluyvera intermedia</i>	0	100	100	65	0	0	0	0	0	89	89	0	65	100	100	100	100	65	100	100	100
<i>Leclercia adecarboxylata</i>	100	100	0	0	0	48	0	0	0	0	79	0	97	93	100	97	93	66	100	86	100
<i>Leminorella grimontii</i>	0	100	0	100	100	0	0	0	0	0	0	0	0	0	100	33	0	0	0	83	0
<i>Leminorella richardii</i>	0	0	0	0	100	0	0	0	0	0	0	0	0	0	100	0	0	0	0	0	0
<i>Moellerella wisconsensis</i>	0	100	0	80	0	0	0	0	0	0	0	0	70	0	100	0	100	100	60	0	0
<i>Morganella morganii</i> biogroup B	100	95	0	0	15	100	100	100	0	80	0	0	90	5	100	93	0	0	0	0	0
<i>Morganella morganii</i> subsp. <i>morganii</i>	95	95	0	0	20	95	95	1	0	95	95	0	98	1	99	90	1	0	0	0	0
<i>Morganella morganii</i> subsp. <i>sibonii</i>	50	86	0	0	7	100	93	29	0	64	79	0	79	0	100	86	0	7	0	0	0
<i>Pantoea agglomerans</i>	20	50	70	50	0	20	20	0	0	0	85	2	35	65	100	20	40	75	100	15	65
<i>Pantoea dispersa</i>	0	82	64	100	0	0	9	0	0	0	100	0	82	9	100	0	0	1	100	0	0
<i>Photorhabdus asymbiotica</i>	0	0	0	20	0	60	0	0	0	0	100	80	20	0	100	0	0	0	0	0	0
<i>Plesiomonas shigelloides</i>	99	97	0	0	17	0	5	99	98	93	90	0	1	0	100	0	70	0	0	0	5
<i>Proteus mirabilis</i>	2	97	50	65	98	98	98	0	0	99	95	90	98	2	100	96	2	15	0	0	0
<i>Proteus myxofaciens</i>	0	100	100	50	0	100	100	0	0	0	100	100	100	0	100	100	0	100	0	0	0
<i>Proteus penneri</i>	0	100	0	0	30	100	99	0	0	0	85	50	99	0	100	45	1	100	0	0	0

Adonitol fermentation	myo-Inositol fermentation	d-Sorbitol fermentation	L-Arabinose fermentation	Raffinose fermentation	L-Rhamnose fermentation	Maltose fermentation	d-Xylose fermentation	Trehalose fermentation	Cellobiose fermentation	α -Methyl-d-glucoside fermentation	Erythritol fermentation	Esculin hydrolysis	Melibiose fermentation	d-Arabitol fermentation	Glycerol fermentation	Mucate fermentation	Tartrate, Jordan's	Acetate utilization	Lipase (Corn oil)	DNase (25°C)	Nitrate nitrite	Oxidase, Kovacs'	ONPG test	Yellow pigment	d-Mannose fermentation
97	55	65	98	90	55	95	95	98	92	70	0	80	97	95	65	25	50	2	0	0	80	0	80	0	100
90	95	99	99	99	99	98	99	99	98	90	0	99	99	98	97	90	95	75	0	0	99	0	99	0	99
100	95	100	100	90	96	100	100	100	100	0	0	30	100	100	50	0	50	0	0	0	100	0	0	0	100
0	0	40	100	98	100	100	99	100	100	98	0	99	99	0	40	90	35	50	0	0	100	0	100	0	100
0	0	45	100	100	100	100	91	100	100	95	0	100	100	0	5	81	19	86	0	0	100	0	100	0	100
0	0	0	100	100	83	100	100	100	100	100	0	100	100	0	33	83	50	83	0	0	100	0	100	0	100
0	0	100	100	100	100	100	100	100	100	100	0	100	100	0	100	100	100	0	0	0	100	0	100	0	100
93	0	0	100	66	100	100	100	100	100	0	0	100	100	96	3	93	83	28	0	0	100	0	100	37	100
0	0	0	100	0	0	0	83	0	0	0	0	0	0	0	17	100	100	0	0	0	100	0	0	0	0
0	0	0	100	0	0	0	100	0	0	0	0	0	0	0	0	50	100	0	0	0	100	0	0	0	0
100	0	0	0	100	0	30	0	0	0	0	0	0	100	75	10	0	30	10	0	0	90	0	90	0	100
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	0	100	0	0	0	90	0	20	0	100
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	95	0	0	0	90	0	10	0	98
0	0	0	0	0	0	0	0	100	0	0	0	0	0	0	7	7	100	0	0	0	100	0	0	0	100
7	15	30	95	30	85	89	93	97	55	7	0	60	50	50	30	40	25	30	0	0	85	0	90	75	98
0	0	0	100	0	91	82	100	100	55	0	0	0	0	100	27	0	9	100	0	0	91	0	91	27	100
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	60	20	0	0	0	0	0	60	100
0	98	0	0	0	0	90	0	100	0	0	0	11	60	0	50	0	50	6	0	0	0	100	99	90	
0	0	0	0	1	1	0	98	98	1	0	0	0	0	0	70	0	87	20	92	50	95	0	0	0	0
0	0	0	0	0	0	100	0	100	0	100	0	0	0	0	100	0	100	0	100	50	100	0	0	0	0
0	0	0	0	1	0	100	100	55	0	80	0	0	0	0	55	0	85	5	45	40	90	0	1	0	0

Continued

Table 19.12 Biochemical reactions of selected named species of the order Enterobacterales^a—cont'd

Organism	Indole production	Methyl red	Voges-proskauer	Citrate (Simmons)	H ₂ S (TSI)	Urea hydrolysis	Phenylalanine deaminase	Lysine decarboxylase	Arginine dihydrolase	Ornithine decarboxylase	Motility	Gelatin hydrolysis (22° C)	Growth in KCN	Malonate utilization	d-Glucose, acid	d-Glucose, gas	Lactose fermentation	Sucrose fermentation	d-Mannitol fermentation	Dulcitol fermentation	Salicin fermentation
<i>Proteus vulgaris</i>	98	95	0	15	95	95	99	0	0	0	95	91	99	0	100	85	2	97	0	0	50
<i>Providencia alcalifaciens</i>	99	99	0	98	0	0	98	0	0	1	96	0	100	0	100	85	0	15	2	0	1
<i>Providencia rettgeri</i>	99	93	0	95	0	98	98	0	0	0	94	0	97	0	100	10	5	15	100	0	50
<i>Providencia rustigianii</i>	98	65	0	15	0	0	100	0	0	0	30	0	100	0	100	35	0	35	0	0	0
<i>Providencia stuartii</i>	98	100	0	93	0	30	95	0	0	0	85	0	100	0	100	0	2	50	10	0	2
<i>Rahnella aquatilis</i>	0	88	100	94	0	0	95	0	0	0	6	0	0	100	100	98	100	100	100	88	100
<i>Raoultella ornithinolytica</i>	100	96	70	100	0	100	0	100	0	100	0	0	100	100	100	100	100	100	100	10	100
<i>Raoultella planticola</i>	20	100	98	100	0	98	0	100	0	0	0	0	100	100	100	100	100	100	100	15	100
<i>Raoultella terrigena</i>	0	60	100	40	0	0	0	100	0	20	0	0	100	100	100	80	100	100	100	20	100
<i>Salmonella bongori</i>	0	100	0	94	100	0	0	100	94	100	100	0	100	0	100	94	0	0	100	94	0
<i>Salmonella enterica</i> subsp. <i>arizonae</i>	1	100	0	99	99	0	0	99	70	99	99	0	1	95	100	99	15	1	100	0	0
<i>Salmonella enterica</i> subsp. <i>diarizonae</i>	2	100	0	98	99	0	0	99	70	99	99	0	1	95	100	99	85	5	100	1	0
<i>Salmonella enterica</i> subsp. <i>enterica</i>	1	100	0	95	95	1	0	98	70	97	95	0	0	0	100	96	1	1	100	96	0
<i>Salmonella enterica</i> subsp. <i>houteanae</i>	0	100	0	98	100	2	0	100	70	100	98	0	95	0	100	100	0	0	98	0	60
<i>Salmonella enterica</i> subsp. <i>indica</i>	0	100	0	89	100	0	0	100	67	100	100	0	0	0	100	100	22	0	100	67	0
<i>Salmonella enterica</i> subsp. <i>salamae</i>	2	100	0	100	100	0	0	100	90	100	98	2	0	95	100	100	1	1	100	90	5
<i>Salmonella</i> sero-type Choleraesuis	0	100	0	25	50	0	0	95	55	100	95	0	0	0	100	95	0	0	98	5	0
<i>Salmonella</i> sero-type Gallinarum	0	100	0	0	100	0	0	90	10	1	0	0	0	0	100	0	0	0	100	90	0
<i>Salmonella</i> sero-type Paratyphi A	0	100	0	0	10	0	0	0	15	95	95	0	0	0	100	99	0	0	100	90	0
<i>Salmonella</i> sero-type Pullorum	0	90	0	0	90	0	0	100	10	95	0	0	0	0	100	90	0	0	100	0	0

Adonitol fermentation	myo-Inositol fermentation	d-Sorbitol fermentation	L-Arabinose fermentation	Raffinose fermentation	L-Rhamnose fermentation	Maltose fermentation	d-Xylose fermentation	Trehalose fermentation	Cellobiose fermentation	α -Methyl-d-glucoside fermentation	Erythritol fermentation	Esculin hydrolysis	Melibiose fermentation	d-Arabitol fermentation	Glycerol fermentation	Mucate fermentation	Tartrate, jordan's	Acetate utilization	Lipase (Corn oil)	DNase (25° C)	Nitrate nitrite	Oxidase, Kovacs'	ONPG test	Yellow pigment	d-Mannose fermentation
0	0	0	0	1	5	97	95	30	0	60	1	50	0	0	60	0	80	25	80	80	98	0	1	0	0
98	1	1	1	1	0	1	1	2	0	0	0	0	0	0	15	0	90	40	0	0	100	0	1	0	100
100	90	1	0	5	70	2	10	0	3	2	75	35	5	100	60	0	95	60	0	0	100	0	5	0	100
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	50	25	0	0	100	0	0	0	100
5	95	1	1	7	0	1	7	98	5	0	0	0	0	0	50	0	90	75	0	10	100	0	10	0	100
0	0	94	100	94	94	94	94	100	100	0	0	100	100	0	13	30	6	6	0	0	100	0	100	0	100
100	95	100	100	100	100	100	100	100	100	100	0	100	100	100	100	96	100	95	0	0	100	0	100	0	100
100	100	92	100	100	100	100	100	100	100	100	0	100	100	100	100	100	100	62	0	0	100	0	100	1	100
100	80	100	100	100	100	100	100	100	100	100	0	100	100	100	100	100	100	20	0	0	100	0	100	0	100
0	0	100	94	0	88	100	100	100	0	0	0	0	94	0	0	88	0	100	0	0	100	0	94	0	100
0	0	99	99	1	99	98	100	99	1	1	0	1	95	1	10	90	5	90	0	2	100	0	100	0	100
0	0	99	99	1	99	98	100	99	1	1	0	1	95	1	10	30	20	75	0	2	100	0	92	0	100
0	35	95	99	2	95	97	97	99	5	2	0	5	95	0	5	90	90	90	0	2	100	0	2	0	100
5	0	100	100	0	98	100	100	100	50	0	0	0	100	5	0	0	65	70	0	0	100	0	0	0	100
0	0	0	100	0	100	100	100	100	0	0	0	0	89	0	33	89	100	89	0	0	100	0	44	0	100
0	5	100	100	0	100	100	100	100	0	8	0	15	8	0	25	96	50	95	0	0	100	0	15	0	95
0	0	90	0	1	100	95	98	0	0	0	1	0	45	1	0	0	85	1	0	0	98	0	0	0	95
0	0	1	80	10	10	90	70	50	10	0	1	0	0	0	0	50	100	0	0	10	100	0	0	0	100
0	0	95	100	0	100	95	0	100	5	0	0	0	95	0	10	0	0	0	0	0	100	0	0	0	100
0	0	10	100	1	100	5	90	90	5	0	0	0	0	0	0	0	0	0	0	0	100	0	0	0	100

Continued

Table 19.12 Biochemical reactions of selected named species of the order Enterobacterales^a — cont'd

Organism	Indole production	Methyl red	Voges-proskauer	Citrate (Simmons)	H ₂ S (TSI)	Urea hydrolysis	Phenylalanine deaminase	Lysine decarboxylase	Arginine dihydrolase	Ornithine decarboxylase	Motility	Gelatin hydrolysis (22° C)	Growth in KCN	Malonate utilization	d-Glucose, acid	d-Glucose, gas	Lactose fermentation	Sucrose fermentation	d-Mannitol fermentation	Dulcitol fermentation	Salicin fermentation
<i>Salmonella</i> sero-type Typhi	0	100	0	0	97	0	0	98	3	0	97	0	0	0	100	0	1	0	100	0	0
<i>Serratia liquefaciens</i>	1	93	93	90	0	3	0	95	0	95	95	90	90	2	100	75	10	98	100	0	97
<i>Serratia marcescens</i>	1	20	98	98	0	15	0	99	0	99	97	90	95	3	100	55	2	99	99	0	95
<i>Serratia odorifera</i> biogroup 1	60	100	50	100	0	5	0	100	0	100	100	95	60	0	100	0	70	100	100	0	98
<i>Serratia odorifera</i> biogroup 2	50	60	100	97	0	0	0	94	0	0	100	94	19	0	100	13	97	0	97	0	45
<i>Serratia plymuthica</i>	0	94	80	75	0	0	0	0	0	0	50	60	30	0	100	40	80	100	100	0	94
<i>Serratia rubidaea</i>	0	20	100	95	0	2	0	55	0	0	85	90	25	94	100	30	100	99	100	0	99
<i>Shigella boydii</i>	25	100	0	0	0	0	0	0	18	2	0	0	0	0	100	0	1	0	97	5	0
<i>Shigella dysenteriae</i>	45	99	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	1	100	0	0
<i>Shigella flexneri</i>	50	100	0	0	0	0	0	0	5	0	0	0	100	0	100	3	1	1	95	1	0
<i>Shigella sonnei</i>	0	100	0	0	0	0	0	0	2	98	0	0	0	0	100	0	2	1	99	0	0
<i>Tatumella ptyseos</i>	0	0	5	2	0	0	90	0	0	0	0	0	0	0	100	0	0	98	0	0	55
<i>Xenorhabdus nematophila</i>	40	0	0	0	0	0	0	0	0	0	100	80	0	0	80	0	0	0	0	0	0
<i>Yersinia enterocolitica</i>	50	97	2	0	0	75	0	0	0	95	2	0	2	0	100	5	5	95	98	0	20
<i>Yersinia pestis</i>	0	80	0	0	0	5	0	0	0	0	0	0	0	0	100	0	0	0	97	0	70
<i>Yersinia pseudotuberculosis</i>	0	100	0	0	0	95	0	0	0	0	0	0	0	0	100	0	0	0	100	0	25
<i>Yersinia ruckeri</i>	0	97	10	0	0	0	0	50	5	100	0	30	15	0	100	5	0	0	100	0	0

^aEach number is the percentage of positive reactions after 2 days of incubation at 36° C unless noted otherwise. Most of these positive reactions occur within 24 hours. Reactions that become positive after 2 days are not considered.

H₂S, Hydrogen sulfide; KCN, potassium cyanide; ONPG, o-Nitrophenyl-β-D-galactopyranoside; TSI, triple sugar iron.

Modified from the Centers for Disease Control and Prevention, Atlanta, GA, and Farmer, J. J., et al. (2006). The genera *Aeromonas* and *Plesiomonas*. In M. Dworkin, et al. (Eds.). *The prokaryotes. A handbook on the biology of bacteria: Proteobacteria: Gamma subclass: Vol. 6* (3rd ed, p. 564). New York: Springer.

Adonitol fermentation	myo-Inositol fermentation	d-Sorbitol fermentation	L-Arabinose fermentation	Raffinose fermentation	L-Rhamnose fermentation	Maltose fermentation	d-Xylose fermentation	Trehalose fermentation	Cellobiose fermentation	α -Methyl-d-glucoside fermentation	Erythritol fermentation	Esculin hydrolysis	Melibiose fermentation	d-Arabitol fermentation	Glycerol fermentation	Mucate fermentation	Tartrate, jordan's	Acetate utilization	Lipase (Corn oil)	DNase (25° C)	Nitrate nitrite	Oxidase, kovacs'	ONPG test	Yellow pigment	d-Mannose fermentation
0	0	99	2	0	0	97	82	100	0	0	0	0	100	0	20	0	100	0	0	0	100	0	0	0	100
5	60	95	98	85	15	98	100	100	5	5	0	97	75	0	95	0	75	40	85	85	100	0	93	0	100
40	75	99	0	2	0	96	7	99	5	0	1	95	0	0	95	0	75	50	98	98	98	0	95	0	99
50	100	100	100	100	95	100	100	100	100	0	0	95	100	0	40	5	100	60	35	100	100	0	100	0	100
55	100	100	100	7	94	100	100	100	100	0	7	40	96	0	50	0	100	65	65	100	100	0	100	0	100
0	50	65	100	94	0	94	94	100	88	70	0	81	93	0	50	0	100	55	70	100	100	0	70	0	100
99	20	1	100	99	1	99	99	100	94	1	0	94	99	85	20	0	70	80	99	99	100	0	100	0	100
0	0	43	94	0	1	20	11	85	0	0	0	0	15	0	50	0	50	0	0	0	100	0	10	0	100
0	0	30	45	0	30	15	4	90	0	0	0	0	0	0	10	0	75	0	0	0	99	0	30	0	100
0	0	29	60	40	5	30	2	65	0	0	0	0	55	1	10	0	30	8	0	0	99	0	1	0	100
0	0	2	95	3	75	90	2	100	5	0	0	0	25	0	15	10	90	0	0	0	100	0	90	0	100
0	0	0	0	11	0	0	9	93	0	0	0	0	25	0	7	0	0	0	0	0	98	0	0	0	100
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	60	0	0	20	20	0	0	60	80
0	30	99	98	5	1	75	70	98	75	0	0	25	1	40	90	0	85	15	55	5	98	0	95	0	100
0	0	50	100	0	1	80	90	100	0	0	0	50	20	0	50	0	0	0	0	0	85	0	50	0	100
0	0	0	50	15	70	95	100	100	0	0	0	95	70	0	50	0	50	0	0	0	95	0	70	0	100
0	0	50	5	5	0	95	0	95	5	0	0	0	0	0	30	0	30	0	30	0	75	0	50	0	100

Table 19.13 Designation of *Salmonella* serogroups of most clinical significance

Old designation	New designation
A	2
B	4
C ₁ -C ₄	6, 7
C ₂ -C ₃	8
D ₁	9
D ₂	9, 46
D ₃	9, 46, 27
E ₁ -E ₂ -E ₃	3, 10
E ₄	1, 3, 19
F	11
G ₁ -G ₂	13

From Grimont, P. A. D., & Weill, F.-X. (2007). *Antigenic formulae of the Salmonella serovars*, 2007. (9th ed.). Paris, France: WHO Collaborating Centre for Reference and Research on *Salmonella*.

POINTS TO REMEMBER

- Most of the genera discussed in this chapter could conceivably be isolated from almost any clinical specimen, especially when dealing with immunocompromised patients.
- Although most isolates of *E. coli* are considered normal fecal microbiota, several strains (enterovirulent *E. coli*) are known to cause intestinal tract infections; in the United States STEC is the most notable. *E. coli* is the most significant cause of UTIs.
- ETEC strains can produce LT and/or ST.
- EIEC strains genetically and phenotypically resemble *Shigella* spp.
- STEC strains produce Shiga toxin 1 and/or Shiga toxin 2. Some of these strains, referred to as EHEC, produce a more severe infection called HUS.
- *Salmonella* and *Shigella* are enteric pathogens and are not considered normal fecal biota.
- *Yersinia pestis*, one of the most virulent species in the order Enterobacterales, causes the extraintestinal infection plague.
- A good patient history combined with proper selective screening agar (e.g., HE, XLD, and SMAC agars) can be very helpful in the timely and accurate identification of enteric pathogens associated with diarrheal disease.
- The use of an initial battery of selective agar media and key biochemical tests that include an oxidase test, TSI, urea, and LIA can often result in a presumptive genus identification that can be confirmed with either conventional biochemical tests, one of several multitest or rapid and automated identification systems, or a molecular biology assay.
- Serogrouping is an important aspect in the identification of enteric pathogens.

LEARNING ASSESSMENT QUESTIONS

- S. dysenteriae*
 - S. flexneri*
- Which of the following test results is most helpful in categorizing an isolate as a member of the family Moraxellaceae?
 - Positive urea
 - Positive Voges-Proskauer
 - Positive phenylalanine deaminase
 - Positive lactose fermentation
- Which agent is most often the cause of transmission of bubonic plague to humans?
 - Fleas
 - Mosquitos
 - Dog bites
 - Inhalation
- A 47-year-old patient who just returned from Mexico was admitted to the hospital with a 3-day history of vomiting and diarrhea, without fever. No fecal leukocytes were found in the stool. A stool culture grew an organism identified as *Escherichia coli*. Which strain is the most likely cause of the infection?
 - EPEC
 - ETEC
 - ESTEC
 - EIEC
- A gram-negative, oxidase-negative bacillus was isolated from the cerebrospinal fluid of an infant in the newborn nursery. The organism produced dark pink colonies on MAC agar, was β -hemolytic on SBA, and had the following biochemical results: spot indole positive, triple sugar iron, acid over acid with gas; urease-negative; and citrate-negative. What is the most probable identity of this organism?
 - Escherichia coli*
 - Klebsiella aerogenes*
 - Klebsiella pneumoniae*
 - Serratia marcescens*
- Which member of the order Enterobacterales is often associated with lobar pneumonia in hospitalized older adults?
 - Shigella* spp.
 - Proteus vulgaris*
 - Escherichia coli*
 - Klebsiella pneumoniae*
- What is the most common cause of community-acquired urinary tract infections?
 - Klebsiella pneumoniae*
 - Escherichia coli*
 - Providencia stuartii*
 - Citrobacter freundii*
- Which organism is an opportunistic pathogen that can cause wound and urinary tract infections and the production of kidney stones?
 - Yersinia enterocolitica*
 - Citrobacter freundii*
 - Proteus mirabilis*
 - Enterobacter cloacae*
- Which of the following is an enteric organism that is acquired by eating improperly prepared and cooked or preserved food that is contaminated with human feces and produces dysentery?
 - Proteus vulgaris*
 - Yersinia enterocolitica*
 - Serratia marcescens*
 - Shigella* spp.

11. An organism isolated from a stool culture produces colorless colonies on MAC agar, green colonies with black centers on Hektoen enteric agar, and K/A H₂S positivity in TSI agar. It is urease negative, motile, and phenylalanine deaminase negative. What is the most likely identification of the organism?
 - a. *Proteus vulgaris*
 - b. *Yersinia enterocolitica*
 - c. *Salmonella* spp.
 - d. *Shigella* spp.
12. Which of the following organisms causes infections that present with watery stools followed by bloody stools, abdominal cramping, low-grade fever, and hemorrhagic diarrhea, especially in children?
 - a. *E. coli* O157:H7
 - b. *Shigella sonnei*
 - c. *Salmonella enterica*
 - d. *Yersinia enterocolitica*
13. A 4-year-old boy is admitted to the hospital because of lower-right quadrant pain and a fever of 102° F. The white blood cell count was 15,000/mm³ with 75% PMN and 6% bands. The presumptive diagnosis is acute appendicitis. At surgery, the appendix is grossly normal, but several enlarged red mesenteric lymph nodes are observed. One of the lymph nodes is excised and sent to the laboratory for culture. Which of the following organisms is most likely to produce this clinical picture?
 - a. *Yersinia enterocolitica*
 - b. *Citrobacter freundii*
 - c. *Proteus mirabilis*
 - d. *Enterobacter cloacae*
14. Which of the following organisms would you most likely recover from an infected gallbladder?
 - a. *Escherichia coli* O157:H7
 - b. *Shigella sonnei*
 - c. *Salmonella* spp.
 - d. *Yersinia enterocolitica*
15. A suspected stool pathogen appears as non motile, gram-negative rods. It is H₂S negative, is oxidase negative, does not produce gas when glucose is fermented, and agglutinates the *Shigella* antisera group D. What is the most likely identification of the organism?
 - a. *S. sonnei*
 - b. *S. boydii*
 - c. *S. dysenteriae*
 - d. *S. flexneri*

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Vibrio, Aeromonas, Campylobacter, and Campylobacter-like species

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CHAPTER OUTLINE

***Vibrio*, 456**

General characteristics, 456
Vibrio cholerae, 458
Vibrio parahaemolyticus, 459
Vibrio vulnificus, 460
Vibrio alginolyticus, 460
 Laboratory diagnosis, 460
 Antimicrobial susceptibility, 463

***Aeromonas*, 463**

General characteristics, 463
 Clinical manifestations, 464
 Laboratory diagnosis, 464
 Antimicrobial susceptibility, 466

***Campylobacter* and *Campylobacter*-like species, 467**

Epidemiology, 467
 Clinical manifestations, 468
 Laboratory diagnosis, 468
 Antimicrobial susceptibility, 471

Bibliography, 472

OBJECTIVES

After reading and studying this chapter, you should be able to:

1. Discuss the habitat in which the organisms discussed in this chapter are found.
2. Describe the colony morphology and microscopic characteristics of each organism.
3. Apply the appropriate specimen, transport, and processing for maximum recovery of each organism.
4. Justify the media of choice for isolation of each organism.
5. Discuss key biochemical reactions that will help differentiate among the various genera and identify different species.
6. Compare each group of organisms with respect to macroscopic and microscopic morphology, and biochemical reactions for identification.

7. Compare the epidemiology and clinical infections of each group of organisms.
8. Compare the confirmatory tests commonly used to identify these isolates.
9. Discuss the criteria used to differentiate the bacterial species that cause gastrointestinal illnesses.
10. Explain the risk factors associated with acquiring infections by these organisms.
11. Describe the disease states associated with each group of organisms.

KEY TERMS

Asiatic cholera
 Cholera toxin
 Choleragen
 “Darting” motility
 El Tor
 Halophilic

Kanagawa phenomenon
 Microaerophilic
 Thiosulfate citrate bile salt sucrose (TCBS) agar
 Type B gastritis
 Urea breath test

Case in point

A 65-year-old Chinese male patient, in obvious shock, was examined in the emergency department for a painful swelling of the left hand. His medical history revealed bilateral knee joint pain and posthepatic cirrhosis. The day before admission, he had pricked his left index finger while selecting shrimp at a fish market. Initially, he noticed only a local reaction in his finger, but after 12 hours the left hand began to swell. He later experienced nausea, vomiting, and diarrhea. The patient was now clammy with a weak pulse, marked swelling, and bullous formation with gangrenous changes apparent on the left hand. A diagnosis of septic shock was made, and antimicrobial therapy was initiated. Blood and wound cultures subsequently grew an oxidase-positive, gram-negative bacillus that produced pink colonies on MacConkey (MAC) agar and green colonies on **thiosulfate citrate bile salt sucrose (TCBS)** agar and required 3% to 6% NaCl for growth. No acceptable identification could be made with a rapid identification system for gram-negative

bacilli, and final identification was made with conventional biochemicals supplemented with 1% NaCl. The patient suffered a cardiac arrest and died 11 hours after admission.

Issues to consider

After reading the patient's case history, consider:

- Various pathogenic organisms associated with aquatic life that cause human infections
- Disease spectrum and states associated with these organisms
- Presentation of clinical signs and symptoms
- Media of choice to aid in a rapid diagnosis
- Key biochemical reactions to presumptively and definitively identify the organism responsible for this infection
- Treatment of choice for this infectious agent

This chapter discusses agents of diarrheal diseases and other infections caused by species of *Vibrio*, *Aeromonas*, *Campylobacter*, and *Helicobacter*. The microscopic characteristics, epidemiology, and clinical infections caused by each organism are discussed. Specimen collection, transport, growth requirements, and key biochemical reactions used for diagnosis are also included in this chapter. This group of organisms is important because some of them, the *Vibrio* spp. in particular, have been associated with large epidemics and pandemics. In addition, *Campylobacter* spp. infection may play a role in Guillain-Barré syndrome (GBS), and *Helicobacter pylori* can cause ulcers and has been linked to gastric carcinoma.

Vibrio

The genus *Vibrio* resides in the family Vibrionaceae, which includes 15 genera. To date, only about 15 species within the family have been found in human clinical specimens: *Vibrio* (13 species), *Photobacterium* (1 species), and *Grimontia* (1 species). *Vibrio hollisae* is now classified in the genus *Grimontia* as *G. hollisae*, whereas *Vibrio damsela* has been reclassified as *Photobacterium damsela*.

At least 150 species of *Vibrio* are recognized. *Vibrio* spp. are commonly found in a wide variety of aquatic environments, including fresh water, brackish or estuarine water, and marine or salt water. They are temperature sensitive in that in temperate climates in which water temperatures exceed 20° C, as in the summer months, vibrios can easily be isolated from water, suspended particulate matter, algae, plankton, fish, and shellfish. However, their numbers decline markedly in the winter months, and they are generally found only in the sediments. Therefore the risk of infection from all *Vibrio* spp. can be reduced by the avoidance of eating raw or undercooked shellfish, particularly in warmer summer months. This is especially pertinent for those who are immunocompromised or have serious underlying liver disease. Additionally, those who are severely immunosuppressed should avoid exposure of wounds to fresh, estuarine, and marine water sources.

Several gastrointestinal and extraintestinal infections, caused by *Vibrio* spp. have been reported. Some reasons for the clinical isolation of this organism include:

- Increased ocean water temperature because of climate change
- Increased travel to coastal or cholera-endemic areas

- Increased consumption of seafood (particularly raw or undercooked)
- Increased use of recreational water facilities, which encourages aquatic exposure
- Larger populations of immunocompromised individuals
- Increased awareness of the existence and significance of these organisms in the clinical microbiology laboratory

Pandemics (worldwide epidemics) of cholera, a devastating diarrheal disease caused by *Vibrio cholerae*, have been documented since 1817.

Researchers estimate that approximately 1.3 to 4.0 million cases of cholera occur each year, with deaths tolls ranging from 21,000 to 143,000 worldwide. Although in 2018, the World Health Organization (WHO) reported a 60% decrease in cholera cases worldwide, the number of cases reported to the WHO remains high over the past few years. In 2020, 323,369 cholera cases resulting in 857 deaths were reported from 24 countries. The discrepancy in these numbers was attributed to limited surveillance systems where cases are not being recorded. Nevertheless, in 2017, the Global Task Force on Cholera Control partners launched a strategy for cholera control that aims to reduce cholera deaths by 90% and to eliminate cholera in as many as 20 countries by 2030. In addition, oral vaccine campaigns have been implemented in endemic areas and in areas experiencing an outbreak. The WHO has prequalified three oral cholera vaccines for use; however, they are not available in the United States.

General characteristics

Clinical manifestations

Vibrio spp. can be isolated from a number of clinical sources, and most species have been implicated in more than one disease process, ranging from mild gastroenteritis to cholera and from simple wound infections to necrotizing fasciitis and fatal septicemia. Table 20.1 lists the various clinical infections associated with *Vibrio* spp. The four *Vibrio* spp. most often encountered in the clinical laboratory are *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, and *V. alginolyticus*.

Because most laboratories, except those in coastal areas, have a fairly low frequency of isolation of *Vibrio* spp., a good medical history is extremely important. Often the best indication of a possible *Vibrio* infection is the presence of recognized risk factors, such as:

- Recent consumption of raw seafood (especially oysters)
- Recent immigration or foreign travel
- Accidental trauma incurred during contact with fresh, estuarine, or marine water or associated products (e.g., shellfish, oyster or clam shells, fishhooks)

Microscopic morphology

Vibrio spp. are asporogenous, gram-negative rods that measure approximately 0.5 to 0.8 µm in diameter by 1.4 to 2.6 µm in length. Most organisms possess polar, sheathed, monotrichous or multitrichous flagella when grown in broth; however, *V. parahaemolyticus* and *V. alginolyticus* have the ability to swarm on solid media because of the production of lateral

flagella. They have been described typically as curved or comma-shaped gram-negative rods, but this morphology is often seen only in the initial Gram stain of the clinical specimen (Fig. 20.1). Vibrios usually appear as small, straight, gram-negative rods, but they can be highly pleomorphic, especially under suboptimal growth conditions.

Table 20.1 Clinical infections associated with *Vibrio*, *Grimontia*, and *Photobacterium* species

Species	Clinical infection	Frequency
<i>V. cholerae</i> O1	Cholera, gastroenteritis, wound infections, bacteremia	Common
<i>V. cholerae</i> O139	Cholera	Common
<i>V. cholerae</i> non-O1	Gastroenteritis, septicemia, brain abscesses, ear infections	Relatively common
<i>Vibrio parahaemolyticus</i>	Gastroenteritis, wound infections	Common
<i>Vibrio vulnificus</i>	Septicemia, wound infections	Common
<i>Vibrio alginolyticus</i>	Wound infections, ear infections, conjunctivitis, respiratory infections, bacteremia	Common
<i>Vibrio mimicus</i>	Gastroenteritis, ear infections	Uncommon
<i>Vibrio fluvialis</i>	Gastroenteritis	Uncommon
<i>Vibrio furnissii</i>	Gastroenteritis	Uncommon
<i>Vibrio cincinnatiensis</i>	Meningitis, bacteremia	Rare
<i>Vibrio metschnikovii</i>	Septicemia, peritonitis	Rare
<i>Vibrio harveyi</i>	Wound infections	Rare
<i>Grimontia holisae</i>	Gastroenteritis	Uncommon
<i>Photobacterium damsela</i>	Wound infections (cellulitis; necrotizing fasciitis), bacteremia	Uncommon

From Tarr C. L., et al. (2015). *Vibrio* and related organisms. In J. H. Jorgensen, et al. (Eds.), *Manual of clinical microbiology* (11th ed., p. 762). Washington, DC: ASM Press.

Physiology

The vibrios are facultatively anaerobic, and all 13 clinically significant species are catalase and oxidase positive and able to reduce nitrate to nitrite, except for *V. metschnikovii*, which is negative for all three tests. Most species are generally susceptible to the vibriostatic compound O/129 (2,4-diamino-6,7-diisopropylpteridine), exhibiting a zone of inhibition to a 150-μg vibriostatic disk on Mueller-Hinton or trypticase soy agar (Fig. 20.2). However, this test is not as valuable with isolates from India and Bangladesh, in which resistance to both 10- and 150-μg disks to O/129 is common in *V. cholerae* isolates.

Most vibrios, including *V. cholerae*, also exhibit a positive string test observed as a mucoid “stringing” reaction after emulsification of colonies in 0.5% sodium deoxycholate. This occurs as a result of the release of deoxyribonucleic acid (DNA) from lysed cells. All species, except for *V. cholerae* and *V. mimicus*, are **halophilic**, or salt-loving, and require the addition of Na⁺ for growth. Vibrios can be differentiated from the similar genera *Aeromonas* and *Plesiomonas* (now classified in the order Enterobacterales, see Chapter 19) by key biochemical and growth requirement characteristics, as shown in Table 20.2.

Antigenic structure

Little is known about the antigenic structure of *Vibrio* except for *V. cholerae*, *V. parahaemolyticus*, and, to a lesser degree, *V. fluvialis* and *V. vulnificus*. Over 200 serogroups of *V. cholerae*

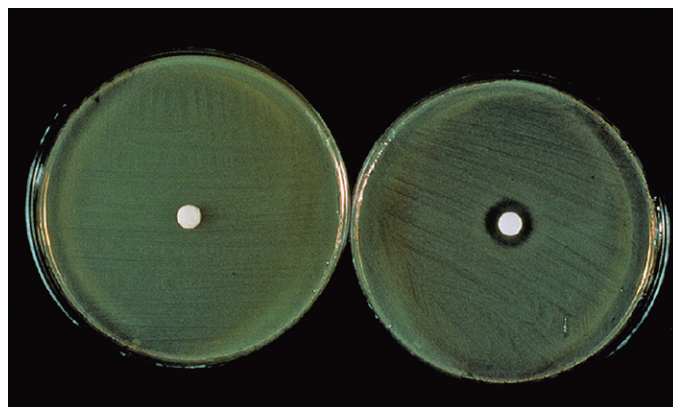


Fig. 20.2 O/129 susceptibility test for *Vibrio* sp. showing a resistant result (left) and a susceptible result (right). (Courtesy J. Michael Janda.)

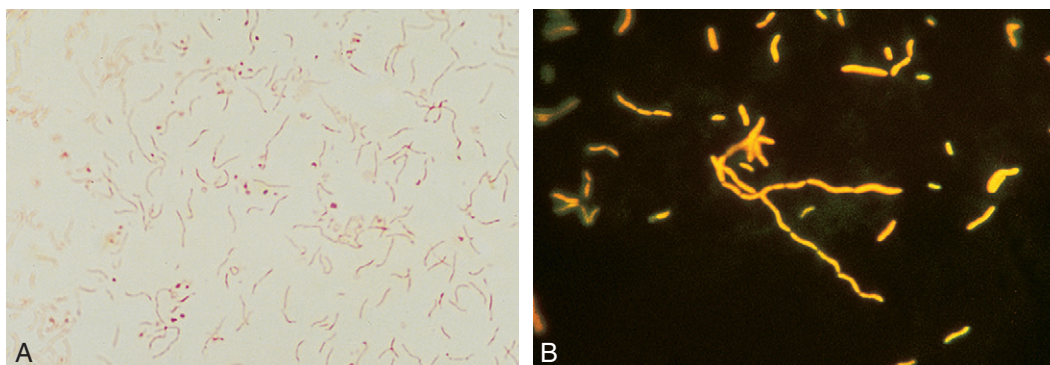


Fig. 20.1 A, Microscopic morphology of *Vibrio* sp. on Gram-stained smear (×1000). B, Acridine orange stain of *Vibrio cholerae* (×1000). (A, Courtesy J. Michael Janda; B, courtesy Rita R. Colwell.)

Table 20.2 Key features for the identification of *Vibrio*, *Aeromonas*, and *Plesiomonas*

Feature	<i>Vibrio</i>	<i>Aeromonas</i>	<i>Plesiomonas</i>
Gram stain reaction	–	–	–
Oxidase activity	+	+	+
O/129 ^a susceptibility			
150 µg	S	R	S
Growth on TCBS agar	+	–	–
0% NaCl	– / +	+	+
6.5% NaCl	+	–	NA
Acid from:			
Glucose	+	+	+
Inositol	–	–	+
Mannitol	+	+	–
Sucrose	+ / –	+ / –	–
Gelatin liquefaction	+	+	–

^aVibriostatic agent (2,4-diamino-6,7-diisopropylpteridine).
 +, most strains positive; –, most strains negative; +/– or – / +, predominant reaction first; NA, not available; R, resistant; S, susceptible; V, variable.
 From Tarr, C. L., et al. (2019). *Vibrio* and related organisms. In K. C. Carroll, et al. (Eds.), *Manual of clinical microbiology* (12th ed., pp. 775–786). Washington, DC: ASM Press.

have been reported. The three major groups of *V. cholerae* are *V. cholerae* O1, *V. cholerae* O139, and *V. cholerae* non-O1, non-O139, all of which share a common flagellar (H) antigen and somatic (O) antigen. Only serogroups O1 and O139 have been responsible for pandemics and large epidemics. Based on the composition of the O antigen, *V. cholerae* O1 organisms are divided into the following serotypes: Ogawa (A, B), Inaba (A, C), and Hikojima (A, B, C; very rarely isolated). Strains that phenotypically resemble *V. cholerae* but fail to agglutinate in O1 antisera are referred to as *V. cholerae* non-O1. *V. parahaemolyticus* can also be serotyped by means of its O and K (capsule) antigens, but this is generally done only during epidemiologic studies conducted by reference laboratories.

Vibrio cholerae

Epidemiology

Vibrio cholerae O1 is the causative agent of cholera, also known as Asiatic cholera or epidemic cholera. It has been a disease of major public health significance for centuries. Most epidemics occur in developing countries, in which it is endemic. In particular, cholera is prevalent in the Bengal region of India and Bangladesh. The seventh and current pandemic of cholera originated in Indonesia in 1961. The pandemic strain, *V. cholerae* O1, spread quickly throughout Asia and reached the African continent in 1970. Cholera returned to South America in 1991 for the first time in almost a century and advanced northward into Central America and even Mexico. In addition, a derivative of the biotype El Tor was responsible for causing a major epidemic in Bangladesh in 1992. In October 2010, a cholera outbreak occurred in Haiti, 10 months after a devastating earthquake. It affected over 820,000 people and killed almost 10,000, and it is considered the worst outbreak

in recent history. The WHO and the Pan American Health Organization (PAHO) reported in January 2020 that the country has achieved 1 year free from confirmed cases of cholera, with the last case reported in January 2019. Cases of cholera are not commonly reported in the United States, and most cases are considered imported cases.

Clinical manifestations

Cholera is an acute diarrheal disease that spreads mainly through contaminated water. However, improperly preserved and handled foods, including fish and seafood, milk, ice cream, and unpreserved meat, have been responsible for outbreaks. The disease manifests in acute cases as a severe gastroenteritis accompanied by vomiting and followed by diarrhea. The stools produced by patients with cholera are described as *rice water stools*, and the number of stools, which are watery and contain numerous flecks of mucus, may be as many as 10 to 30 per day. If left untreated, cholera can result in a rapid fluid and electrolyte loss that leads to dehydration, hypovolemic shock, metabolic acidosis, and death in a matter of hours.

The devastating clinical scenario is the result of a powerful enterotoxin known as **cholera toxin**, or **cholera toxin**. Once ingested, the bacteria colonize the small intestine, in which they multiply and produce cholera toxin. The toxin consists of two toxic A subunits and five binding B subunits. The toxin initially binds to the GM₁ ganglioside receptor on the cell membrane via the B subunits. The A2 subunit then facilitates the entrance of the A1 subunit. Once inside the cell, the active A1 subunit stimulates the production of adenylate cyclase through the inactivation of G protein. This leads to an accumulation of cyclic adenosine monophosphate (cAMP) along the cell membrane, which stimulates hypersecretion of electrolytes (Na⁺, K⁺, HCO₃[–]) and water out of the cell and into the lumen of the intestine, as shown in Fig. 20.3. The net effect is that the gastrointestinal tract's absorptive ability is overwhelmed, resulting in the massive outpouring of watery stools.

Treatment and management of cholera are best accomplished by the administration of copious amounts of intravenous or oral fluids to replace fluids lost from the severe diarrhea. The administration of antimicrobial agents such as doxycycline can shorten the duration of diarrhea and thereby reduce fluid loss; however, resistance to doxycycline has been reported. Therefore administration of additional antimicrobials such as azithromycin and ciprofloxacin may be necessary.

Epidemic *V. cholerae* O1 strains occur in two biogroups, classic and **El Tor**. El Tor has been the predominant biogroup in the past two pandemics. Studies in Bangladesh, however, indicate a rapidly occurring reemergence of the classic biogroup. In addition, new variants have emerged in Africa and Asia. The El Tor biogroup differs from the classic biogroup in that El Tor is Voges-Proskauer positive, hemolyzes erythrocytes, is inhibited by polymyxin B (50 µg), and is able to agglutinate chicken red blood cells. The two biogroups also have different bacteriophage susceptibility patterns.

Numerous cases of *Vibrio* infections have been reported involving other serogroups, *V. cholerae* non-O1. Most of these strains are phenotypically similar to toxigenic *V. cholerae* O1, but most lack the cholera toxin gene and appear to cause a milder form of gastroenteritis or cholera-like disease. However, *V. cholerae* serogroups O75 and O141 harbor the cholera toxin gene and have been associated with sporadic,

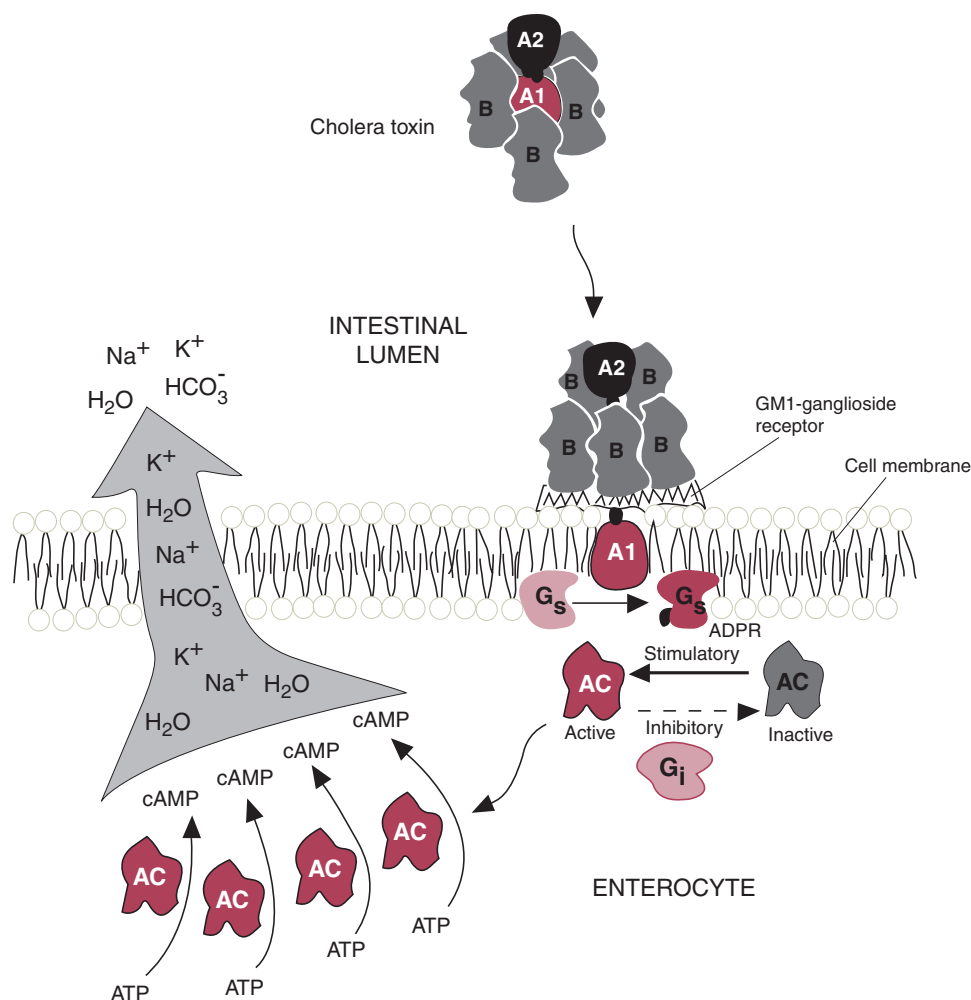


Fig. 20.3 The action of cholera toxin. The complete toxin is shown binding to the GM1 ganglioside receptor on the cell membrane via the binding (B) subunits. The active portion (A1) of the A subunit catalyzes the adenosine diphosphate ribosylation (ADPR) of the G_s (stimulatory) regulatory protein, “locking” it in the active state. Because the G protein acts to return adenylate cyclase from its active to inactive form, the net effect is persistent activation of adenylate cyclase. The increased adenylate cyclase activity results in accumulation of cyclic adenosine 3', 5'-monophosphate (cAMP) along the cell membrane. The cAMP causes the active secretion of sodium (Na⁺), chloride (Cl⁻), potassium (K⁺), bicarbonate (HCO₃⁻), and water out of the cell into the intestinal lumen. ATP, Adenosine triphosphate. (From Ryan, K. J. [2004]. *Vibrio and Campylobacter*. In J. Sherris (Ed.), *Medical microbiology: an introduction to infectious diseases* [4th ed., p. 375]. New York: McGraw-Hill.)

cholera-like diarrhea and bloodstream infections in the United States. Other non-O1 serogroup strains have been implicated in a variety of extraintestinal infections, including epidural brain abscesses and septicemia. The emergence of *V. cholerae* serogroup O139 in 1992 led to a widespread occurrence of cholera cases throughout India and Bangladesh. This was the first *V. cholerae* non-O1 strain producing epidemic disease. The O139 strain contains the cholera toxin gene, *ctx*, and produces a disease that closely resembles cholera caused by *V. cholera* O1. Of interest is that some of these O139 strains share cross-reacting antigens with *Aeromonas trota*, a somewhat uncommon cause of diarrheal disease (discussed later).

Vibrio parahaemolyticus

Epidemiology

Vibrio parahaemolyticus is the second most common *Vibrio* species implicated in gastroenteritis after *V. cholerae*. In the United States, however, it is the most frequently encountered

species in clinical samples. This species was first recognized as a pathogen in Japan in 1950, when it caused a large food poisoning outbreak. Even today it is the primary cause of so-called *summer diarrhea* in Japan. *V. parahaemolyticus* has also been isolated in Europe, the Baltic area, Australia, Africa, Canada, and almost every coastal state in the United States. Most cases of *V. parahaemolyticus* reported before 1996 were associated with various serotypes; however, after 1996, a pandemic strain of *V. parahaemolyticus* serotype O3:K6 emerged and has since been implicated in numerous foodborne outbreaks in various parts of the world.

Like other vibrios, *V. parahaemolyticus* is found in aquatic environments but appears to be limited to coastal or estuarine areas, despite a halophilic requirement of 1% to 8% NaCl. *V. parahaemolyticus* has an association with at least 30 different marine animal species, including oysters, clams, crabs, lobsters, scallops, sardines, and shrimp. Hence, most cases of gastroenteritis can be traced to recent consumption of raw, improperly cooked, or recontaminated seafood, particularly oysters.

In 2019, the Centers for Disease Control and Prevention (CDC) reported a multistate outbreak of gastrointestinal illness linked to oysters imported from Mexico. A total of 16 individuals from 5 states became ill after ingesting the contaminated food. Two people were hospitalized; however, no deaths were reported.

Clinical manifestations

The gastrointestinal disease caused by *V. parahaemolyticus* is generally self-limiting. Patients have watery diarrhea, moderate cramps or vomiting, and low, if any, fever. Symptoms begin about 24 to 48 hours after ingestion of contaminated seafood. *V. parahaemolyticus* has occasionally been isolated from extraintestinal sources such as wounds, ear and eye infections, and even cases of pneumonia. Invariably the patient has a history of recent aquatic exposure or a water-associated traumatic injury to the infected site.

The pathogenesis of *V. parahaemolyticus* is not as well understood as *V. cholerae*. However, there is a possible association between hemolysin production and virulence, known as the **Kanagawa phenomenon**. It has been observed that most clinical *V. parahaemolyticus* strains produce a heat-stable hemolysin that is able to lyse human erythrocytes in a special, high-salt mannitol medium (Wagatsuma agar). These strains are considered Kanagawa toxin positive, whereas most environmental isolates are Kanagawa toxin negative. There are exceptions to both observations, however, and the exact role of this hemolysin in pathogenesis is still not understood. It is also important to note the recent emergence of atypical urease-positive *V. parahaemolyticus* strains from clinical sources along the Pacific coast of North America.

Vibrio vulnificus

Vibrio vulnificus can be found in marine environments on the Atlantic, Gulf, and Pacific coasts of North America. After cholera, the second most serious types of *Vibrio*-associated infections are those caused by *V. vulnificus*. Infections caused by *V. vulnificus* generally fall into two categories, primary septicemia and wound infections. The former is thought to occur through the gastrointestinal route after the consumption of shellfish, especially raw oysters. Patients with liver dysfunction and syndromes that result in increased serum levels of iron (e.g., hemochromatosis, cirrhosis, thalassemia major, hepatitis) are particularly predisposed to this scenario. Within hours, septicemia can develop, with a mortality rate above 50%. Starting in 2007, the CDC required that cases of *V. vulnificus* be reported to local and state public health departments.

Patients with *V. vulnificus* wound infections invariably have a history of some type of traumatic aquatic wound that often presents as a cellulitis. Such cases have also been documented as progressing to necrotizing fasciitis and/or

multiple organ system failure. Although such infections can occur in healthy hosts, the most serious cases are in immunocompromised individuals and those who have experienced a mild to severe injury to the infected site. Infections acquired from wound infections have a 20% to 30% fatality rate. One such case involved a fatal episode of multiple organ dysfunction, only 7 days after a 58-year-old Canadian patient developed septicemia with *V. vulnificus* after merely handling raw tilapia fish he had purchased for dinner.

Vibrio alginolyticus

Of the four major *Vibrio* spp. likely to be encountered in the clinical laboratory, *V. alginolyticus* is the least pathogenic for humans but is frequently isolated in clinical samples in the United States. It is a common inhabitant of marine environments and is a strict halophile, requiring at least 1% NaCl; it is able to tolerate up to 10% NaCl. Almost all isolates originate from extraintestinal sources, such as eye and ear infections or wound and burn infections. The organism can be an occupational hazard for people in constant contact with seawater, such as fishermen or sailors.

Laboratory diagnosis

Specimen collection and transport

Patients' history, including travel, underlying health conditions, recent consumption of seafood or contact with marine water, and gastroenteritis with cholera-like or rice-water stools, is important in diagnosing vibriosis. Vibrios are not fastidious, and only a few special collection and processing procedures are necessary to ensure the recovery from clinical material. Stool specimens should be collected as early as possible in the course of the illness and preferably before the administration of antimicrobial agents. Whenever possible, body fluids, pus, or tissues should be submitted, but swabs are acceptable if they are transported in an appropriate holding medium, such as Cary-Blair, to prevent desiccation. Buffered glycerol saline is not recommended as a transport or holding medium because glycerol is toxic for vibrios. Strips of blotting paper soaked in liquid stool and placed in airtight plastic bags with a few drops of saline to maintain moisture are considered viable specimens for up to 5 weeks.

Culture media

The salt concentration (0.5%) in most commonly used laboratory media, such as nutrient agar or sheep blood agar (SBA), is sufficient to support the growth of many vibrios. On SBA or chocolate (CHOC) agar, vibrios produce medium to large colonies that appear smooth, opaque, and iridescent with a greenish hue. The SBA plate should also be examined for the presence of α - or β -hemolysis. On MAC agar, the pathogenic vibrios usually grow as nonlactose fermenters. However, lactose-fermenting species such as *V. vulnificus* may be overlooked and incorrectly considered to be members of the order Enterobacterales, such as *Escherichia coli*. It is therefore imperative to determine the oxidase activity of any suspicious *Vibrio*-like colony. This can be accomplished by directly testing colonies from SBA or CHOC agar plates with oxidase

Case check 20.1

The patient in the Case in Point had a typical case history and clinical signs and symptoms of *V. vulnificus* infection. The patient suffered from posthepatic cirrhosis, a predisposing factor. In addition, he had suffered a minor wound after handling shellfish that rapidly developed into septic shock.

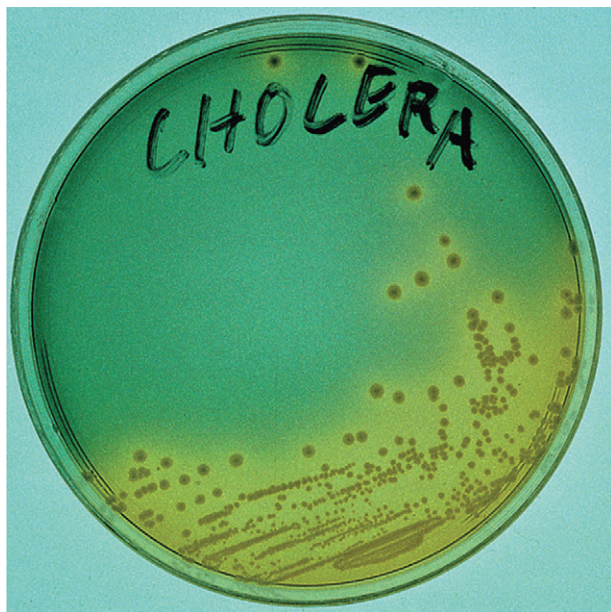


Fig. 20.4 *Vibrio cholerae* on thiosulfate citrate bile salt sucrose agar. (Courtesy S.W. Joseph.)

reagent or by subculturing any suspicious, lactose-fermenting colonies on MAC to an SBA plate for next-day testing. This is necessary because lactose-positive colonies from selective differential media, such as MAC agar, can give false-positive oxidase reactions.

If a selective medium is warranted because of the clinical history (exposure to seafood or seawater) or for geographic reasons (coastal area resident or recent foreign travel), TCBS agar is recommended. It differentiates sucrose-fermenting (yellow colonies) species (Fig. 20.4), such as *V. cholerae*, *V. alginolyticus*, *V. fluvialis*, *V. furnissii*, *V. cincinnatiensis*, *V. metschnikovii*, and some *V. vulnificus* strains, from the nonsucrose-fermenting (green) species, such as *V. mimicus*, *V. parahaemolyticus*, *P. damsela*, and most *V. vulnificus* strains. About 50% of *V. harveyi* isolates are sucrose-positive. Although TCBS generally inhibits all other organisms, it should be monitored with stringent quality control measures. There is great variation among different lots in regard to performance, and not all *Vibrio* spp. grow on TCBS, especially *Grimontia* (formerly *Vibrio*) *hollisae*.

If an enrichment procedure is desired to enhance isolation of vibrios, alkaline peptone water with 1% NaCl (pH 8.5) can be inoculated (at least 20 mL) and incubated for 5 to 8 hours at 35° C then subcultured at 6 hours and 18 hours onto TCBS medium. CHROMagar *Vibrio* (CHROMagar Microbiology, Paris) for the isolation and identification of *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* is available and can be used in

detecting these species in environmental samples and seafood. *Vibrio* colonies appear from white to pale blue to violet in color.

Presumptive identification

Several key tests can aid in the initial identification of a *Vibrio* isolate. Vibrios can be easily confused with other genera, including *Aeromonas*, *Plesiomonas*, *Pseudomonas*, and members of the order Enterobacterales. Their general susceptibility to the vibriostatic agent O/129 (150 µg) and positive string test distinguishes them from *Aeromonas*. Inability to ferment inositol (except for *V. cincinnatiensis* and some strains of *V. metschnikovii*) separates them from *Plesiomonas*. Their positive oxidase reaction (except for *V. metschnikovii*) separates them from the Enterobacterales (excluding *Plesiomonas shigelloides*), and carbohydrate fermentation metabolism separates them from the oxidative *Pseudomonas*.

Definitive identification

Numerous useful biochemical tests aid in the definitive identification of most isolates to the species level. Table 20.3 outlines 8 key differential tests to divide the 10 most clinically significant *Vibrio* spp. into 6 groups as an initial identification step. Some additional tests necessary to identify eight clinical *Vibrio* spp. from groups 1, 5, and 6 are summarized in Table 20.4. It is important to note that with the halophilic vibrios, it often is necessary to add at least 1% NaCl to most biochemical media to obtain reliable reaction results.

Rapid and semiautomated identification systems

Although rapid and semiautomated identification systems contain databases of the more commonly encountered clinical vibrios, they generally are inadequate for accurate identification of these species, particularly those that are less commonly encountered. The inoculating suspensions should contain at least 0.85% NaCl, such as that found with the API-20E identification strips (bioMérieux Vitek, Hazelwood, MO). Even then, the halophilic species might grow poorly, if at all, and they often are confused with other genera, such as *Aeromonas*. Identification using commercial systems remains challenging. One study evaluated six commercially available systems that resulted in identification of only 63% to 81% of organisms at the species level. Most authorities advocate identification with conventional biochemicals, supplemented with additional NaCl, if warranted; alternatively, the rapid system identification should be confirmed at a reference laboratory or state public health department laboratory.

The use of 16S ribosomal ribonucleic acid (rRNA) sequencing alone is less than ideal because of small differences in sequences and polymorphisms among the various species. As databases are developed, matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry might become a rapid, cost-effective means of identification. This method has demonstrated accurate identification of *V. parahaemolyticus*.

Serology

Agglutination, vibriocidal, or antitoxin tests performed on acute and convalescent sera are considered reliable methods for testing vibrios serologically. In any case, all isolates

Case check 20.2

It is important to use selective media and key biochemical reactions to differentiate *Vibrio* from *Vibrio*-like organisms. Key biochemical tests to differentiate various *Vibrio* spp. include reaction on TCBS, agent O/129 susceptibility or resistance, the string test, and growth in different concentrations of NaCl. The Case in Point uses the results obtained from these reactions to identify the responsible organism.

Table 20.3 Key differential tests for the 6 groups of 12 *Vibrio* species that occur in clinical specimens^a

	Group 1		Group 2	Group 3	Group 4	Group 5			Group 6			
	<i>V. cholerae</i>	<i>V. mimicus</i>	<i>V. metschnikovii</i>	<i>V. cincinnatiensis</i>	<i>G. hollisae</i>	<i>P. damsela</i>	<i>V. fluvialis</i> ^b	<i>V.furnissii</i>	<i>V. alginolyticus</i>	<i>V. parahaemo- lyticus</i>	<i>V. vulnificus</i>	<i>V. harveyi</i>
Growth in nutrient broth with:												
No NaCl added ^c	+	+	—	—	—	—	—	—	—	—	—	—
6 % NaCl added	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase production	+	+	—	+	+	+	+	+	+	+	+	+
Nitrate reduced to nitrite	+	+	—	+	+	+	+	+	+	+	+	+
<i>Myo</i> -Inositol fermentation	—	—	V	+	—	—	—	—	—	—	—	—
Arginine dihydrolase	—	—	V	—	—	+	+	+	—	—	—	—
Lysine decarboxylase	+	+	V	V	—	V	—	—	+	+	+	+
Ornithine decarboxylase	+	+	—	—	—	—	—	—	V	+	V	—

^aShowing reactions of these species in different groups. All data except those for oxidase production and nitrate reduction are for reactions that occur within 2 days at 35° to 37° C; oxidase production and nitrate reduction reactions are done only at day 1.

^bIncludes *V. furnissii*, which differs from *V. fluvialis* primarily by production of gas in D-glucose.

^cSpecies that require salt should have salt added to each biochemical tested.

+, 90% to 100% positive; —, negative, 0% to 10% positive; NG, no growth, possibly because the NaCl concentration is too low, even when 1% NaCl is added; V, variable, 11% to 89% positive. See Table 20.4 for the exact percentages.

From Tarr, C. L., et al. (2019). *Vibrio* and related organisms. In K. C. Carroll, et al. (Eds.), *Manual of clinical microbiology* (12th ed., p. 777). Washington, DC: ASM Press.

Table 20.4 Key differential biochemicals to separate *Vibrio* species in groups 1, 5, and 6^a

	Group 1			Group 5			Group 6		
	<i>V. cholerae</i>	<i>V. mimicus</i>	<i>P. damsela</i>	<i>V. fluvialis</i>	<i>V. furnissii</i>	<i>V. alginolyticus</i>	<i>V. parahaemolyticus</i>	<i>V. vulnificus</i>	<i>V. harveyi</i>
Voges-Proskauer (1% NaCl)	75	9	95	0	0	95	0	0	50
Motility	99	98	25	70	89	99	99	99	0
Acid production from:									
Sucrose	100	0	5	100	100	99	1	15	50
Cellobiose	8	0	0	30	11	3	5	99	50
Salicin	1	0	0	0	0	4	1	95	0

^aNumbers indicate the percentages of strains that are positive after 48 hours of incubation at 36° C (unless other conditions are indicated). Most of the positive reactions occur during the first 24 hours.

From Tarr, C. L., et al. (2019). *Vibrio* and related organisms. In K. C. Carroll, et al. (Eds.), *Manual of clinical microbiology* (12th ed., p. 777). Washington, DC: ASM Press.

with a presumptive biochemical identification of *V. cholerae* should be promptly reported to the appropriate public health authorities, and the isolate should be forwarded to the health department or a reference laboratory for serotyping.

Antimicrobial susceptibility

Both Mueller-Hinton agar and broth contain sufficient salt to support the growth of *Vibrio* spp. most often isolated from clinical specimens. The recommended antimicrobial susceptibility testing methods are standardized disk diffusion (Kirby-Bauer) or dilution susceptibility testing methods. The Clinical and Laboratory Standards Institute (CLSI) has interpretive guidelines only for *V. cholerae* limited to ampicillin, tetracyclines, folate pathway inhibitors, and chloramphenicol. In general, most strains of *V. cholerae* are susceptible to doxycycline or ciprofloxacin. Increased resistance from the acquisition of plasmids is relatively uncommon in the United States, but there are reports of multiple resistant strains from Asia and Africa.

Because most vibrios grow rather rapidly and are similar to enteric bacteria in many ways, it is often useful for a first approximation to use the interpretive guidelines for the Enterobacterales when testing *Vibrio* isolates other than *V. cholerae* for agents not currently covered by the CLSI documents M100 (*V. cholerae*) and M45 (other species). In general, fluoroquinolones alone or the synergistic combination of ciprofloxacin and cefotaxime display excellent in vitro activity against *V. vulnificus* strains. Most vibrios are also susceptible to gentamicin, tetracyclines, chloramphenicol (except *P. damsela*), monobactams, carbapenems, and fluoroquinolones.

Aeromonas

The genus *Aeromonas* consists of ubiquitous, oxidase-positive, glucose-fermenting, gram-negative rods (Fig. 20.5) that are widely distributed in freshwater, estuarine, and marine environments worldwide. They are frequently isolated from retail produce sources and animal meat products. Aeromonads are responsible for a diverse spectrum of disease syndromes in warm- and cold-blooded animals, including fish, reptiles, amphibians, mammals, and humans.

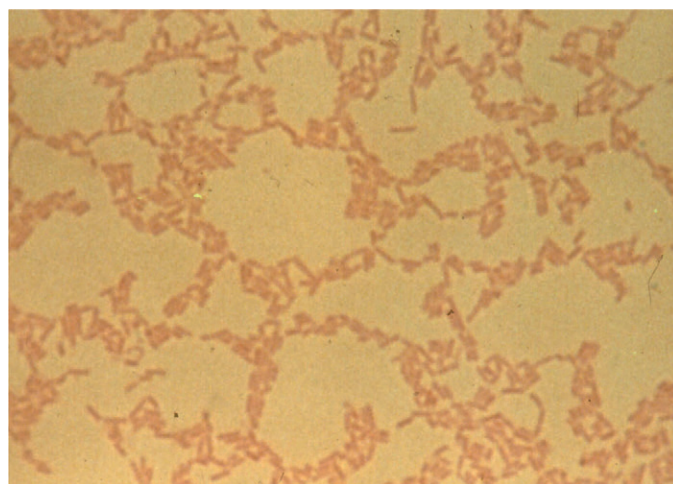


Fig. 20.5 Microscopic morphology of *Aeromonas* on Gram-stained smear (×1000). (Courtesy J. Michael Janda.)

Previously, the genus *Aeromonas* resided in the family Vibrionaceae. However, phylogenetic evidence from molecular studies resulted in the proposal of a separate family, Aeromonadaceae. Currently, there are 36 species of aeromonads characterized in the latest edition of *Bergey's manual of systematic bacteriology*. Of those species, at least 19 have been identified as emerging pathogens in humans. A recent study, however, reported most human pathogens (95.4%) were identified from only four species: *Aeromonas caviae* (37.26%), *Aeromonas dhakensis* (23.49%), *Aeromonas veronii* (21.54%), and *Aeromonas hydrophila* (13.07%). In the United States, aeromonads have a seasonal pattern of increased isolation from May to October, reflecting their increased numbers in aquatic environments in the warmer months.

General characteristics

Aeromonads are straight rods (1.0 to 3.5 μm long by 0.3 to 1.0 μm wide). In the mid 1970s, they were classified into one of two groups, the mesophilic group that causes several human infections (motile organisms with optimal growth around 37° C) or the psychrophilic group that is linked to fish diseases

(nonmotile strains with optimal growth around 22° C). The mesophilic group consists of three different complexes or groups of species. One is the *A. hydrophila* complex, which includes *A. hydrophila* subsp. *hydrophila*, *A. hydrophila* subsp. *ranae*, *A. bestiarum*, and *A. dhakensis*. Another is the *A. veronii* complex, which includes *A. veronii* biovar *sobria* (formerly misidentified as *A. sobria*), *A. veronii* biovar *veronii*, *A. jandaei*, *A. trota*, *A. schubertii*, *A. diversa*, and *A. encheleia*. The last mesophilic complex is the *A. caviae* complex, which includes the species *A. caviae*, *A. media*, *A. rivipollensis*, and *A. eucrenophila*. These organisms are considered mesophiles because they grow well at 37° C. They are all motile by means of a single polar flagellum.

The psychrophilic group consists of only one species, *A. salmonicida*, which is a fish pathogen with several subspecies. This organism is nonmotile and grows best at 22° to 25° C. Psychrophilic nonmotile strains are not considered human pathogens. All aeromonads, in general, can typically grow from 10° to 42° C.

Clinical manifestations

Intestinal infections

The aeromonads are recognized as enteric pathogens, albeit not in the same manner as the more common enteric pathogens *Salmonella*, *Shigella*, and *V. cholerae*. The level and pattern of virulence is more like the multifactorial patterns of the various *E. coli* subgroups associated with enteric disease (e.g., enterotoxigenic *E. coli*, enterohemorrhagic *E. coli*). Therefore screening of stool specimens for the presence of these organisms, followed by further identification to the species level, is appropriate. This is especially true in cases of pediatric diarrhea or in any immunocompromised individual.

The medical history of patients displaying diarrhea and harboring aeromonads often, but not always, involves aquatic exposure, such as an association with untreated groundwater or consumption of seafood, particularly raw oysters or clams. Infections have also been linked to fresh produce and dairy products. Five diarrheal presentations are observed in patients in whom *Aeromonas* has been isolated from their stools:

1. Acute, secretory diarrhea often accompanied by vomiting
2. An acute, dysenteric form of diarrhea (similar to shigellosis) with blood and mucus
3. Chronic diarrhea usually lasting more than 10 days
4. A cholera-like disease, including rice-water stools
5. The nebulous syndrome commonly referred to as *traveler's diarrhea* (similar to enterotoxigenic *E. coli*). Most cases are self-limiting, but in pediatric, geriatric, and immunocompromised populations, supportive therapy and antimicrobials are often indicated.

A. caviae is the species most frequently associated with gastrointestinal infections, especially in neonate and pediatric populations; it has been associated with inflammatory bowel disease. Other species associated with diarrhea include *A. hydrophila* and *A. veronii* (biovars *sobria* and *veronii*). More serious complications, usually from infections with *A. hydrophila* and *A. veronii* biovar *sobria*, include hemolytic uremic syndrome or kidney disease that might require kidney transplantation. *A. veronii* biovar *sobria* has also been

linked to cholera-like disease characterized by abdominal pain, fever, and nausea.

Extraintestinal infections

Aeromonads are also responsible for extraintestinal infections; septicemia and wound infections are the most common. They have also been implicated in cases of meningitis, osteomyelitis, pelvic abscesses, otitis, cystitis, endocarditis, peritonitis, cholecystitis, keratitis associated with contact lens wear, and endophthalmitis in healthy and immunocompromised individuals. Wound infection invariably involves a recent traumatic aquatic exposure, such as a boating or fishing accident, and generally occurs on the extremities. The most common presentation is cellulitis, although there have been a few cases of myonecrosis and necrotizing fasciitis and even a rare case of ecthyma gangrenosum associated with sepsis. Aeromonad wound isolates are predominantly *A. hydrophila* subsp. *hydrophila*, *A. veronii*, and *A. dhakensis*. Among the survivors of the 2004 tsunami in southern Thailand, *Aeromonas* spp. were the most common cause of skin and soft tissue infections. Elevated numbers of *Aeromonas* spp. were recorded in flood water samples in New Orleans after Hurricane Katrina in 2005.

An association between *A. veronii* biovar *sobria* and surgical wound infections involving the use of leeches for medicinal therapy after plastic surgery to relieve venous congestion has been noted. These patients can develop serious aeromonad wound infections. It appears that the leech *Hirudo medicinalis* has a symbiotic relationship with this aeromonad species within its gut, wherein the organisms aid in the enzymatic digestion of the blood ingested by the leech.

Aeromonad sepsis is one of the most invasive type of *Aeromonas* infection and similarly has a strong association with the species *A. veronii* biovar *sobria*, *A. caviae*, *A. dhakensis*, and *A. hydrophila*. Such patients are most likely to be immunocompromised and have a history of liver disease or dysfunction, hematologic malignancies, hepatobiliary disorders, or traumatic injuries. Also at high risk are individuals with leukemia, lymphoma, or myeloma. Although the original source of infection is often unknown, it is surmised that it is the gastrointestinal, biliary, or even more rarely, respiratory tract.

Laboratory diagnosis

Culture media

Aeromonads grow readily on most media used for routine and stool cultures. After 24-hour incubation at 35° C, aeromonads appear as large, round, raised, opaque colonies with an entire edge and a smooth, often mucoid, surface. Frequently, an extremely strong odor is present, and pigmentation ranges from translucent and white to buff-colored. Hemolysis is variable on SBA, but most major clinical species, such as *A. hydrophila*, *A. veronii* biovar *sobria*, and *A. jandaei*, display strong β -hemolysis (Fig. 20.6). In addition, the most commonly isolated species, *A. caviae*, has been showing increasing incidence of β -hemolysis on SBA. Although aeromonads grow on almost all enteric media, they often are overlooked on MAC agar, especially in stool cultures, because a number of aeromonads ferment lactose. This is of particular

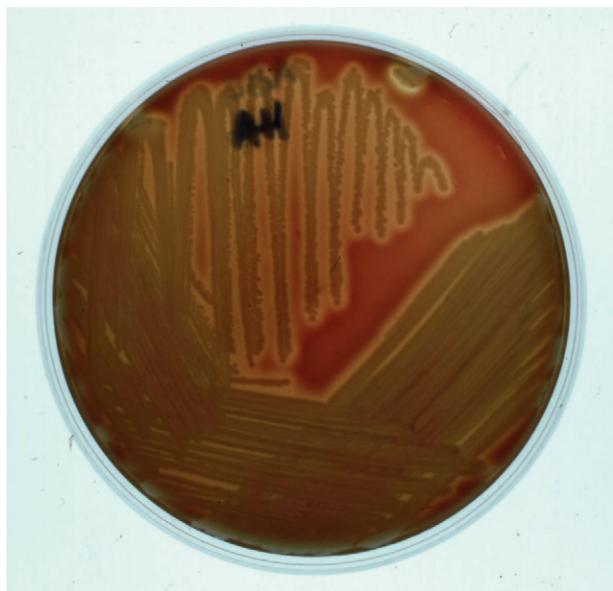


Fig. 20.6 *Aeromonas hydrophila* exhibiting β -hemolysis on sheep blood agar. (Courtesy A. J. Horneman.)

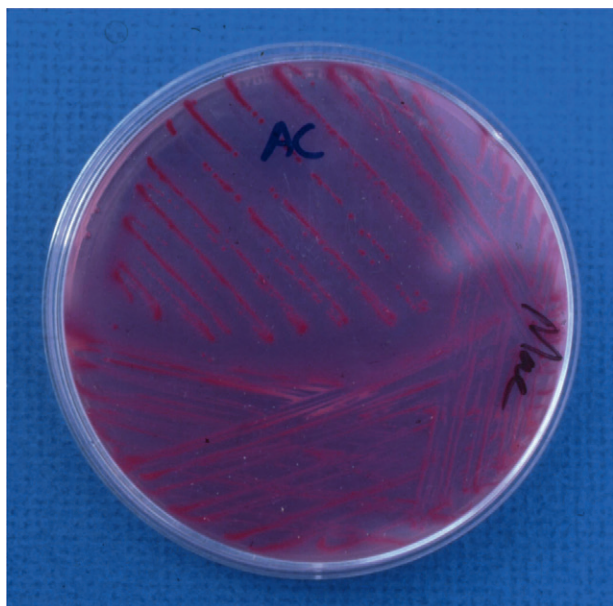


Fig. 20.7 *Aeromonas caviae* exhibiting lactose fermentation on MacConkey agar. (Courtesy A. J. Horneman.)

concern because the most commonly isolated species, especially in pediatric cases of diarrhea, is the lactose-fermenting *A. caviae*. This particular species is generally overlooked as normal biota *E. coli* (Fig. 20.7).

The combined use of SBA with 20 μ g of ampicillin per milliliter and a modified cefsulodin-irgasin-novobiocin (CIN) plate, with only 4 μ g of cefsulodin instead of 15 μ g, might yield the highest recovery of aeromonads. However, the incorporation of ampicillin in the blood agar may inhibit some *A. caviae* as well as all *A. trota* strains because the hallmark feature of *A. trota* is its unusual universal susceptibility to ampicillin. Therefore SBA without ampicillin is preferred. On the standard CIN formulation for enteric *Yersinia* or the

modified CIN media, *Aeromonas* will form pink-centered colonies from the fermentation of mannitol, with an uneven, clear apron resembling *Yersinia enterocolitica*. However, an oxidase test performed on SBA colonies will easily separate the oxidase-positive aeromonads from the oxidase-negative yersinias. The use of an enrichment broth is generally not considered necessary. However, if such a medium is warranted for detecting chronic cases or asymptomatic carriers, alkaline peptone water is recommended. This can be inoculated, incubated overnight at 35° C, and subsequently subcultured to appropriate plate media.

Presumptive identification

An important screening procedure for aeromonads is to perform an oxidase test and a spot indole test on suspicious colonies on SBA, especially β -hemolytic colonies. A positive oxidase test distinguishes aeromonads from the order Enterobacterales (except for *Plesiomonas shigelloides*), and most clinically relevant aeromonads are indole-positive. The presence and type of hemolysis among multiple aeromonad colony types in a single culture often are the only clues to an infection involving more than one species of *Aeromonas*.

Members of the genera *Vibrio*, *Aeromonas*, and *Plesiomonas* have many similar characteristics. See Table 20.2 for key tests to help separate these three genera. The best tests to distinguish the aeromonads from *Vibrio* spp. are the string test (usually negative for aeromonads and positive for vibrios) and testing for sensitivity to O/129 (aeromonads are usually resistant, and most vibrios are susceptible; see Fig. 20.2).

A test to separate aeromonads and plesiomonads from most vibrios is determining the ability to grow in the presence of NaCl. Aeromonads and plesiomonads grow well in nutrient broth with 0% NaCl, but not in 6% NaCl. Conversely, most vibrios, specifically the halophilic species, cannot grow in 0% NaCl but thrive in 6% NaCl and even higher concentrations of NaCl (Fig. 20.8). However, because *V. cholerae* and *V. mimicus* are nonhalophilic and grow well without additional salt, any salt tolerance test must be used in conjunction with the string test and O/129 disk to distinguish aeromonads from this major pandemic cholera species and the less common, sucrose-negative *V. mimicus*. Fermentation of inositol can be used for separating aeromonads from plesiomonads, in which aeromonads are negative and plesiomonads are positive. Finally, the ability to ferment glucose, with or without the production of gas, distinguishes *Aeromonas* from oxidase-positive, nonfermenting *Pseudomonas* isolates.

Definitive identification

Nucleic acid amplification tests (NAATs) are the most accurate in the identification of aeromonads. Definitive identification is also accomplished with a small number of conventional and readily available biochemical tests, including the API-20E test strip (bioMérieux); however, limitations in identifying aeromonads to the species level exist with most commercially available systems. MALDI-TOF mass spectrometry accurately identifies most aeromonads to the genus level, but identification to the species level is limited. Two FDA-cleared systems are available: the MALDI Biotyper

system (Bruker, Billerica, MA) and the IVD 3.0 Vitek MS (bioMérieux), but again, limitations occur in both systems because of limited database content. Typing systems such as PFGE and PCR-based techniques are also available for identification. Serologic assays (i.e., immunoblotting and

enzyme-linked immunosorbent assay [ELISA]) yield low sensitivity and are not readily dependable.

Conventional biochemical tests as shown in Table 20.5 are useful in the identification of aeromonads to the species level; however, they have the longest turnaround time of all methods described with the lowest performance.

Antimicrobial susceptibility

Although most cases of *Aeromonas*-associated gastroenteritis are self-limiting, antimicrobial therapy is often indicated. It is always warranted in wound infections and septicemia. As a genus, aeromonads are almost uniformly resistant to penicillin, ampicillin, and cephalothin or cefazolin, except for the susceptibility of *A. trota* to ampicillin. Aeromonads are variable with regard to ticarcillin or piperacillin and cefoxitin.

Thus far, *A. veronii* biovar *veronii* isolates still appear to be susceptible to cephalothin, but only *A. veronii* biovar *veronii* is 100% susceptible to cefoxitin. Otherwise, aeromonads are generally susceptible to trimethoprim-sulfamethoxazole, aminoglycosides, and quinolones, although *A. veronii* biovar *veronii* is moderately resistant to tobramycin. It should be noted that there have been reports of a case of sepsis with an extended-spectrum β -lactamase (ESBL)-producing *A. hydrophila* and a case of necrotizing fasciitis, with the probable in vivo transfer of a TEM-24 plasmid-borne gene coding for an ESBL from *Enterobacter aerogenes*.

CLSI antimicrobial susceptibility studies should always be determined by the standard Kirby-Bauer disk diffusion method. This is because of possible serious discrepancies in β -lactamase detection by rapid minimal inhibitory concentration methods that would directly affect the proper treatment of aeromonad infections.

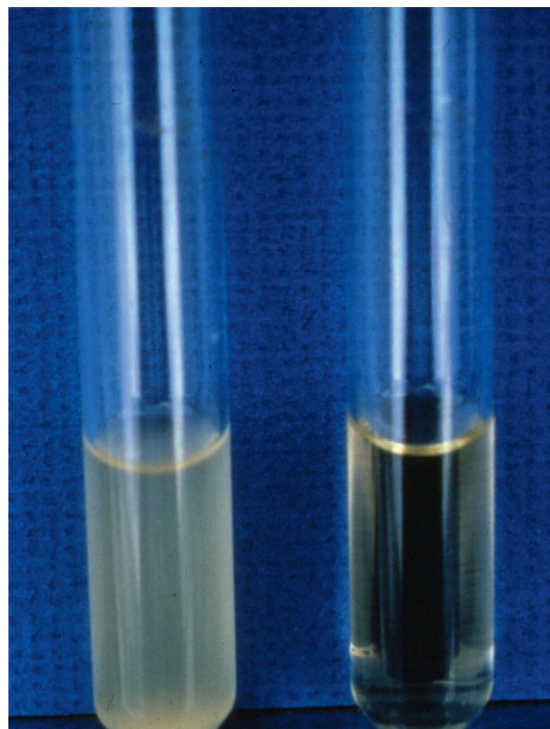


Fig. 20.8 Both *Aeromonas* and *Plesiomonas* spp. will grow in nutrient broth with 0% NaCl (left), whereas neither will grow in nutrient broth with 6% NaCl (right). (Courtesy A. J. Horneman.)

Table 20.5 Differential characteristics for clinical *Aeromonas* species

Characteristic	Aeromonas species						
	<i>A. hydrophila</i>	<i>A. veronii</i> bio- var <i>sobria</i>	<i>A. veronii</i> biovar <i>veronii</i>	<i>A. caviae</i>	<i>A. schubertii</i>	<i>A. jandaei</i>	<i>A. trota</i>
Esculin hydrolysis	+	–	+	+	–	–	–
Voges-Proskauer	+	+	+	–	–	+	–
Pyrazinamidase activity	V	ND	ND	+	ND	ND	ND
Fermentation							
Arabinose	+	–	–	+	–	–	–
Cellobiose	–	V	V	+	–	V	+
Mannitol	+	+	+	+	–	+	V
Sucrose	+	+	+	+	–	–	V
Susceptibility							
Ampicillin	R	R	R	R	R	R	S
Ticarcillin or piperacillin	V	V	V	V	V	V	S
Cephalothin	–R	=S	S	–R	S	–R	R
Glucose (gas)	+	+	+	–	–	+	V

+, Positive; –, negative; ND, not determined; R, resistant; S, susceptible; V, variable.

From Lamy, B., & Horneman, A. J. (2019). *Aeromonas*. In K. C. Carroll, et al. (Eds.), *Manual of clinical microbiology* (12th ed., p. 771). Washington, DC: ASM Press.

Case check 20.3

Although *Aeromonas* and *Plesiomonas* are similar to *Vibrio* spp., the key biochemical results used in the Case in Point were able to rule out *Aeromonas* and *Plesiomonas* as the infecting organism and aided in definitively identifying *Vibrio* as the pathogen causing the disease.

Campylobacter and Campylobacter-like species

Campylobacter and *Campylobacter*-like species, which include *Helicobacter*, *Arcobacter*, and *Wolinella*, have undergone changes in taxonomy. Campylobacters were formerly classified with the vibrios because of their positive oxidase and characteristic microscopic morphology, but DNA homology studies showed that *Campylobacter* spp. do not belong with the vibrios. In addition, unlike the vibrios, which are fermentative, most campylobacters are asaccharolytic. The genus *Campylobacter* belongs to the family Campylobacteraceae, whereas the genera *Helicobacter* and *Wolinella* are members of the family Helicobacteriaceae. The genus *Acrobacter* is in the family Arcobacteraceae.

Based on rRNA sequence studies, *Wolinella recta* and *Wolinella curva* were transferred to the genus *Campylobacter* as *C. rectus* and *C. curvus*. Although they may appear to be strict anaerobes, they have been grown in a **microaerophilic** environment. Microaerophilic organisms, such as *Campylobacter*, require oxygen, but at a concentration less than that of room air; 5% is normally optimal. To date, there are 43 species of *Campylobacter*. There are 39 species of *Helicobacter*, most of which colonize the stomachs of mammals.

Epidemiology

Campylobacter spp. have been known to cause abortion in domestic animals, such as cattle, sheep, and swine, and are primarily zoonotic organisms. Although these organisms were suspected of causing human infections earlier, campylobacters were not established as human pathogens until sensitive isolation procedures and media were developed. Today, the most common cause of bacterial gastroenteritis worldwide is *Campylobacter jejuni*. The transmission of campylobacteriosis has been attributed to direct contact with animals and handling infected pets, such as dogs, cats, and birds, and indirect contact by consumption of contaminated water and dairy products and improperly cooked poultry. Person-to-person transmission has been reported, and some *Campylobacter* spp. are also sexually transmitted.

In 2019, the Foodborne Diseases Active Surveillance Network estimated that approximately 20 cases of campylobacteriosis are diagnosed every year for each 100,000 people in the population. The CDC reported 236 foodborne outbreaks of *Campylobacter* from 2010 to 2017, accounting for 2381 illnesses. Poultry, raw milk, and untreated water were the sources most often identified. Because of unreported or misdiagnosed cases, it is believed campylobacteriosis affects over 1.5 million individuals each year. The populations that most often manifest the disease include infants and young adults, although all age groups are at risk. In addition to

C. jejuni, *C. coli* and *C. lari* also cause gastrointestinal disease (the enteric campylobacters).

Campylobacter fetus subsp. *fetus* has been isolated most frequently from blood cultures and is rarely associated with gastrointestinal illness. Most infections occur in immunocompromised individuals, during pregnancy, and in older adults. *Arcobacter* spp. have been isolated from a variety of animals: cattle, pigs, sheep, birds, etc. In humans, they have been linked to bacteremia, endocarditis, peritonitis, and diarrhea. Table 20.6 summarizes the clinical significance of *Campylobacter*, *Arcobacter*, and *Helicobacter* organisms.

Table 20.6 Clinically significant *Campylobacter* species and *Campylobacter*-like organisms

Species	Clinical Significance
<i>Arcobacter butzleri</i>	Associated with diarrheal disease and bacteremia in humans and in children with recurring gastrointestinal illness (abdominal cramps), endocarditis, and peritonitis
<i>Arcobacter cryaerophilus</i>	Isolated from cases of human bacteremia and diarrhea
<i>Arcobacter skirrowii</i>	Gastroenteritis
<i>Campylobacter jejuni</i>	Most common cause of bacterial diarrhea worldwide
<i>Campylobacter coli</i>	Gastroenteritis, bacteremia in immunocompromised patients
<i>Campylobacter fetus</i> subsp. <i>fetus</i>	Bacteremia in immunocompromised patients and extraintestinal infections during pregnancy
<i>Campylobacter concisus</i>	Involved in periodontal disease; has also been recovered from individuals with gastrointestinal illness
<i>Campylobacter curvus</i>	Gastroenteritis, periodontal disease
<i>Campylobacter gracilis</i>	Bacteremia, soft tissue abscesses
<i>Campylobacter hyointestinalis</i>	Gastroenteritis
<i>Campylobacter lari</i>	Enteritis very similar to that caused by <i>Campylobacter jejuni</i> , also septicemia
<i>Campylobacter rectus</i>	Gastroenteritis; associated with periodontal disease
<i>Campylobacter sputorum</i> biovar <i>sputorum</i>	Lung, axillary, groin abscesses
<i>Helicobacter pylori</i>	Common cause of duodenal ulcers and type B gastritis; risk factor in gastric carcinoma, and MALT lymphoma
<i>Helicobacter bilis</i>	Bacteremia, sepsis
<i>Helicobacter canadensis</i>	Gastroenteritis
<i>Helicobacter cinaedi</i>	Colitis, cellulitis, and sepsis
<i>Helicobacter felis</i>	Gastritis and ulcers
<i>Helicobacter fennelliae</i>	Colitis, sepsis

Helicobacter pylori is strongly associated with gastric, peptic, and duodenal ulcers and with gastrointestinal carcinoma. *H. pylori* has been identified in as many as 80% of gastric ulcer patients. Although the organism was previously found in human gastric tissue, it was difficult to assess their significance because the samples were taken at autopsy. It is estimated that 50% of the population worldwide is infected with *H. pylori*. In developing countries in Africa, Asia, and South America, the incidence is reported to be as high as 80% to 90%. This higher incidence is attributed to poor sanitary conditions, and it appears that infection occurs early in life. Some data suggest human-to-human transmission and the possibility of human reservoirs. Although it is not conclusively proven, fresh groundwater is the likely source of many infections.

Clinical manifestations

Campylobacter

Several *Campylobacter* spp. have been implicated in human infection, the most common being *C. jejuni*, *C. coli*, *C. fetus* subsp. *fetus*, *C. lari*, and *C. upsaliensis*. Patients infected with *C. jejuni* present with a diarrheal disease that begins with mild abdominal pain within 2 to 10 days after ingestion of the organism. Cramps and diarrhea (with or without blood/fecal white cells) often follow the initial signs and symptoms. Patients may experience fever and chills and, rarely, nausea and vomiting. In most patients, the illness is self-limiting and usually resolves in 2 to 6 days. Untreated patients can remain carriers for several months. Other enteric *Campylobacter* infections (those caused by *C. coli*, *C. lari*, and *C. upsaliensis*) have similar clinical manifestations.

The *C. fetus* species contains three subspecies, *C. fetus* subsp. *fetus*, *C. fetus* subsp. *venerealis*, and *C. fetus* subsp. *testudinum*. *C. fetus* subsp. *fetus* is primarily linked to bacteremia in pregnant women and patients who are immunocompromised. It is also associated with gastroenteritis. However, cases are underreported because this species does not grow at 42° C, the temperature stool cultures are generally incubated to recover the enteric campylobacters.

Strong evidence suggests that *Campylobacter* infection plays a role in GBS, an autoimmune disorder characterized by acute paralysis caused by damage to the peripheral nervous system. Many patients with GBS test positive for antibodies to *Campylobacter*, with an estimated 1 in 1000 individuals diagnosed following a *Campylobacter* infection. It is believed that antibodies produced during a *Campylobacter* infection bind to gangliosides found on peripheral nerves. Cross-reactivity with these nerve cells in an autoimmune response may be responsible for this debilitating nerve disorder.

Helicobacter pylori

H. pylori has been primarily linked to gastric infections and peptic ulcer disease. Once acquired, *H. pylori* colonizes the stomach for a long time and can cause a low-grade inflammatory process, producing a chronic superficial gastritis. Although it does not invade the gastric epithelium, the infection is recognized by the host immune system, which initiates an antibody response. The antibodies produced are not protective, however.

H. pylori is also recognized as a major cause of **type B gastritis**, a chronic condition formerly associated primarily with stress and chemical irritants. In addition, there is a strong association between long-term *H. pylori* infection and gastric cancer. *H. pylori* causes a rare stomach cancer called *mucosa-associated lymphoid tissue* (MALT). This rare type of cancer is the only cancer that may be cured with antimicrobial agents. Other species of helicobacters, including *H. cinaedi* and *H. fennelliae*, have been associated with human gastroenteritis and bacteremia, generally in immunocompromised patients. In addition, enterohepatic helicobacters infect humans and inhabit the intestinal and hepatobiliary tracts of various mammals and birds. It is believed that transmission of these enterohepatic species occurs from animals to humans.

Laboratory diagnosis

Specimen collection and transport

C. fetus subsp. *fetus* can be recovered in several routine blood culture media and is often associated with bacteremia and extraintestinal infections. *Campylobacter* spp. that cause enteric illness are isolated from stool samples and rectal swabs, the less preferred specimen. If a delay in processing the stool specimen is anticipated, it can be placed in a transport medium such as Cary-Blair to maintain the viability of the organisms, modified Cary-Blair, or Fecal Enteric Plus. A common stool transport medium, buffered glycerol saline, is toxic to enteric campylobacters and should therefore be avoided.

H. pylori can be recovered from gastric biopsy materials. Samples must be transported quickly to the laboratory. Stuart medium can be used to maintain the viability of the organisms if a delay in processing is anticipated. Tissue samples may also be placed in cysteine-Brucella broth with 20% glycerol and frozen at -70° C or Portagerm pylori media (bioMérieux).

Culture media

An enriched selective agar, *Campylobacter* blood agar (*Campy* blood agar) is a commonly used medium to isolate *C. jejuni* and other enteric campylobacters. This commercially available medium contains Brucella agar base, 10% sheep red blood cells, and a combination of antimicrobials: vancomycin, trimethoprim, polymyxin B, amphotericin B, and cephalothin. Other selective media that have been successful in recovering *Campylobacter* spp. are Butzler medium and Skirrow's medium. Table 20.7 shows the composition of each of these selective media.

Campy-CVA (cefoperazone-vancomycin-amphotericin B) medium has been reported to provide better suppression of fecal biota, even when this medium is incubated at 37° C. Incubation at 37° C allows the recovery of *Campylobacter* spp. that are inhibited at 42° C. *C. fetus* subsp. *fetus*, *C. rectus*, and *C. curvus* can be isolated using routine culture media. Charcoal-based, blood-free media, such as charcoal cefoperazone deoxycholate agar (CCDA) and charcoal-based selective media (CSM), are also available. Studies have shown that blood-free, charcoal-containing selective medium is superior in isolating *Campylobacter* spp. compared to media containing blood. A combination of media that contains either CCDA or CSM can achieve the highest yield of *Campylobacter* spp.

Table 20.7 Selective media for the cultivation of *Campylobacter* species

Medium	Base	Antimicrobial agent
Campy blood agar plate	Brucella agar	Vancomycin
		Trimethoprim
	10% sheep red blood cells	Polymyxin B
		Amphotericin B
Skirrow's	Heart infusion	Cephalothin
		Vancomycin
	Lysed, defibrinated horse red blood cells	Trimethoprim
		Polymyxin B
Butzler	Columbia blood	Bacitracin
		Novobiocin
	Horse blood, defibrinated	Cycloheximide
		Colistin
CCDA	Nutrient agar	Cefazolin
		Cefoperazone
	Charcoal	Amphotericin B
	Sodium desoxycholate	

CCDA, Charcoal cefoperazone deoxycholate agar (Becton Dickinson, Franklin Lakes, NJ).

in stool samples. To recover *H. pylori*, a combination of a nonselective medium, such as CHOC agar or Brucella agar with 5% horse red blood cells, and a selective medium, such as Skirrow's agar or Wilkins-Chalgren agar, may be used. It is important that the inoculated medium be fresh and moist and that the culture be incubated in a microaerophilic environment, with increased humidity.

Incubation

There is a double purpose for incubating stool cultures at 42° C to recover *C. jejuni*. First, *C. jejuni* and other enteric campylobacters grow optimally at 42° C. Second, growth of colon microbiota is inhibited at this higher temperature. *C. fetus* subsp. *fetus*, on the other hand, is a rare stool isolate, and growth is suppressed at 42° C; therefore to isolate this organism, media should be incubated at 37° C.

Enteric *Campylobacter* and *Helicobacter* spp. require a microaerophilic and capnophilic environment. The ideal atmospheric environment for these organisms is a gas mixture of 5% O₂, 10% CO₂, and 85% N₂ for *Campylobacter* spp. and 4% O₂, 5% CO₂, 5% H₂, and 86% N₂ for *Helicobacter* spp. Except for *C. rectus* and *C. curvus*, a strict anaerobic environment does not support the growth of most *Campylobacter* spp. Several methods can be used to obtain the required environment for campylobacters. With the GasPak EZ Gas Generating Container System (BD Diagnostic Systems, Sparks, MD), specimen plates along with a sachet are placed into a clear plastic incubation container, sealed, and incubated at the appropriate temperature. The sachet is activated on exposure to air once the foil pouch is removed. The GasPak

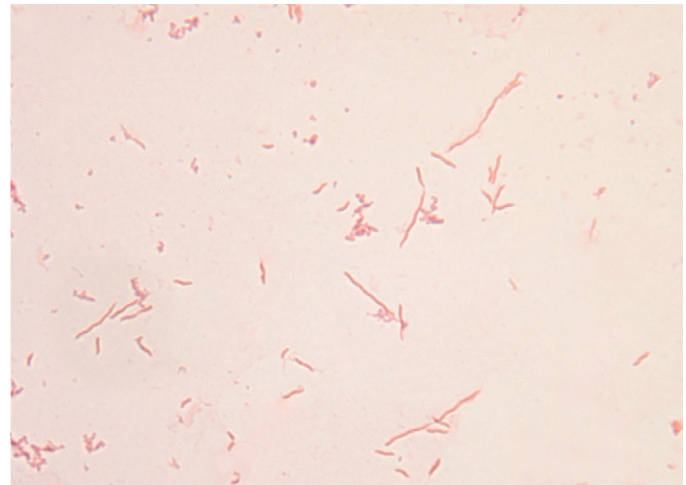


Fig. 20.9 Microscopic morphology of *Campylobacter* on Gram-stained smear (×1000).

EZ Gas Generating Pouch System can hold up to four plates but requires at least two plates per resealable pouch. One sachet is added to each pouch and is activated on exposure to air, similar to the previous procedure. For optimal results, a paper towel or moistened cotton ball with 5mL of water should be placed inside the pouch. The pouch is sealed by closing the zipper part of the pouch and then incubated at the appropriate temperature.

Presumptive identification

Microscopic morphology

Campylobacter spp. are curved, non-spore-forming, gram-negative rods that measure approximately 0.2 to 0.9 μm × 0.5 to 5.0 μm (Fig. 20.9). Enteric campylobacters may appear as long spirals or S- or seagull-wing shapes. These organisms may appear as coccobacilli in smears prepared from older cultures. On Gram-stained smears, these organisms stain poorly. For better visualization, carbol-fuchsin is recommended as a counterstain; if safranin is used, counterstaining should be extended to 2 to 3 minutes.

Campylobacter spp. exhibit a characteristic “darting” motility on hanging drop preparations or when visualized under phase-contrast microscopy. It should be noted that the sensitivity using phase-contrast microscopy to observe motile organisms has not been widely studied; therefore considerable skill is needed to identify these organisms. To observe the typical motility, organisms should be suspended in *Brucella* or tryptic soy broth. Distilled water and saline seem to inhibit motility.

Arcobacter spp. have a microscopic morphology similar to that of *Campylobacter* spp. Unlike the single polar flagellum of campylobacters, *Helicobacter* spp. have either a single polar flagellum or multiple flagella at one pole.

Colony morphology

The typical colony morphology of *C. jejuni* and other enteric campylobacters is moist, runny looking, and spreading. Colonies are usually nonhemolytic; some are round and raised, and others may be flat. *C. fetus* subsp. *fetus* produces smooth, convex, translucent colonies. A tan or slightly pink

Table 20.8 Biochemical tests to differentiate *Campylobacter*, *Arcobacter*, and *Helicobacter* species

Species	Catalase	Nitrate reduction	Urease	H ₂ S production (TSI)	Hippurate hydrolysis	Indoxyl acetate hydrolysis	Growth at			Susceptibility to	
							15° C	25° C	42° C	Nalidixic acid (30µg)	Cephalothin (30µg)
<i>Campylobacter jejuni</i> subsp. <i>jejuni</i>	+	+	–	–	+	+	–	–	+	+	–
<i>Campylobacter jejuni</i> subsp. <i>doylei</i>	V	–	–	–	V	+	–	–	–	+	+
<i>Campylobacter coli</i>	+	+	–	V	–	+	–	–	+	+	–
<i>Campylobacter lari</i>	+	+	V	–	–	–	–	–	+	–	–
<i>Campylobacter fetus</i> subsp. <i>fetus</i>	+	+	–	–	–	–	–	+	–	–	+
<i>Campylobacter upsaliensis</i>	–	+	–	–	–	+	–	–	+	+	+
<i>Campylobacter concisus</i>	–	+	–	V	–	–	–	–	+	–	–
<i>Campylobacter curvus</i>	–	+	–	V	V	V	–	–	+	+	ND
<i>Campylobacter rectus</i>	V	+	–	–	–	+	–	–	W	+	ND
<i>Arcobacter butzleri</i>	V	+	–	–	–	+	+	+	–	V	V
<i>Helicobacter pylori</i>	+	–	+	–	–	–	–	–	–	R	S
<i>Helicobacter fennelliae</i>	+	–	–	–	–	+	–	–	–	S	S
<i>Helicobacter cinaedi</i>	+	+	–	–	–	–	–	–	–	S	I

+, Positive result; –, negative result; I, intermediate; ND, not determined; R, resistant; S, susceptible; TSI, triple sugar iron agar slant; V, variable result; W, weak.

From Nachamkin, I. (2019). *Campylobacter* and *Arcobacter*. In K. C. Carroll, et al. (Eds.), *Manual of clinical microbiology* (12th ed., p. 1032). Washington, DC: ASM Press; and Couturier, M. R. (2019). *Helicobacter*. In K. C. Carroll, et al. (Eds.), *Manual of clinical microbiology* (12th ed., p. 1046). Washington, DC: ASM Press.

coloration is observed in some enteric campylobacter colonies. Other *Campylobacter* species produce colonies similar to those of *C. jejuni*. Although most do not produce pigment, *C. mucosalis* and *C. hyointestinalis* can produce a dirty yellow pigment.

Definitive identification

Isolates from stool specimens and rectal swabs can be presumptively identified as *Campylobacter* spp. by a positive-oxidase, the characteristic Gram-stained microscopic morphology, and the characteristic motility. The microscopic morphology is important because it differentiates *Campylobacter* from other bacteria, such as *Aeromonas* and *Pseudomonas*, which are oxidase-positive and can grow at 42° C in a microaerophilic environment. A positive hippurate hydrolysis is an important characteristic for the identification of *C. jejuni*. Table 20.8 lists the biochemical tests most useful for definitively identifying the most commonly encountered *Campylobacter*, *Arcobacter*, and *Helicobacter* species. *Campylobacter* identification can be achieved using

fecal antigen detection methods and NAATs, including real-time PCR, Prodesse ProGastro SSCS (Hologic GenProbe, San Diego, CA), Luminex xTAG GPP (Luminex Molecular Diagnostics), Verigene EP (Luminex Molecular Diagnostics), BioFire Film Array GP (bioMérieux), and BD Max EB (Becton Dickinson, Franklin Lakes, NJ) assays. Typing systems such as PFGE and multilocus sequence typing (MLST) are also available. MALDI-TOF mass spectrometry can be used to identify common and uncommon species of *Campylobacter*; however, growth conditions must be optimized for successful identification of *Campylobacter* spp.

Helicobacter infections usually are identified by nonculture methods. *H. pylori* can be presumptively identified in a gastric biopsy specimen by a rapid urease test (Fig. 20.10). The collected tissue sample is placed onto Christensen's urea medium and incubated at 37° C for 2 hours. A color change suggests the presence of *H. pylori*. Other rapid, commercially available tests include the CLOtest Rapid Urease Test (Kimberly Clark/Ballard Medical Products, Neenah, WI) and a paper strip test by PyloriTek (BARD, Murray Hill, NJ).



Fig. 20.10 Urease reactions. *Left to right*, Negative, weak positive, positive.

Urease activity can also be detected by the **urea breath test**, which is reportedly sensitive and specific and is recommended for monitoring therapy. In this test, the patient is given ^{13}C -labeled urea orally. Urea degraded by the urease activity of *H. pylori* in the stomach releases $^{13}\text{CO}_2$, which is absorbed into the bloodstream and detected in the exhaled breath by a scintillation counter. *H. pylori* infection can also be diagnosed by fecal antigen detection using enzyme immunoassay methodology, microscopic examination of stained gastric tissue, and DNA amplification tests (e.g., PCR assay).

Immunologic assays

Latex agglutination tests are available for the rapid identification of colonies of enteric campylobacters on primary isolation media. Two commercial kits are available in the United States for culture identification, Campy JCL (Scimedx, Denville, NJ) and DrySpot Campylobacter test kit (Thermo Fisher Scientific, Waltham, MA). These kits can detect the presence of *C. jejuni*, *C. coli*, *C. upsaliensis*, *C. lari*, and sometimes *C. fetus* subsp. *fetus*. However, neither system differentiates among the *Campylobacter* spp. Commercial kits are also available for detecting *Campylobacter* antigen in fecal samples.

Specific antibodies in serum can be detected by ELISA or indirect immunofluorescent assay methods. These methods have been reported to be reasonably sensitive and specific indicators of *Campylobacter* and *H. pylori* infections. Serologic testing is useful for epidemiologic studies for *Campylobacter* but is not recommended for routine diagnosis. Serologic testing measuring IgG antibodies using ELISA kits is an important screening method for the diagnosis of *H. pylori* infection.

Antimicrobial susceptibility

C. jejuni and *C. coli* exhibit variable susceptibilities to antimicrobials such as macrolides, fluoroquinolones,

aminoglycosides, chloramphenicol, and tetracycline. Both species are usually susceptible to macrolides; however, it must be noted that erythromycin resistance rates have increased and are more common in *C. coli* compared to *C. jejuni*. Most patients usually recover without antimicrobial intervention. Ciprofloxacin and other quinolones can also be used; however, ciprofloxacin resistance is high among isolates in the United States. Ampicillin, aminoglycosides, imipenem, and chloramphenicol can be used to treat systemic *C. fetus* infections.

The standard triple therapy for treating *H. pylori* infections consists of clarithromycin, either amoxicillin or metronidazole, and a proton pump inhibitor. An alternative regimen consisting of fluoroquinolone, levofloxacin, and a rifamycin (rifabutin) can be used.

POINTS TO REMEMBER

- Most species in this chapter are found in fresh, estuarine, or marine water.
- Thirteen species of *Vibrio* have been implicated in human infection, and most are agents of diarrheal disease.
- *V. cholerae* produces a powerful enterotoxin and is responsible for large numbers of epidemics and pandemics.
- Other *Vibrio* spp. are common causes of diarrheal disease related to the consumption of raw shellfish or related to aquatic wound infections with serious sequelae, such as septicemia and death.
- TCBS agar is the medium of choice to isolate and differentiate the *Vibrio* spp. This medium distinguishes sucrose-fermenting strains from nonsucrose-fermenting strains.
- *Aeromonas* spp. can cause several types of diarrhea and a variety of extraintestinal infections that can lead to septicemia, meningitis, and death.
- Human *Campylobacter* spp. are generally responsible for gastroenteritis; *C. jejuni* is one of the most common causes of bacterial diarrhea worldwide.
- Patients suffering from GBS often test positive for *Campylobacter* antibodies. GBS is believed to be an autoimmune disorder resulting from cross-reactivity of *Campylobacter* antibodies with the nerve ganglia.
- *H. pylori* is strongly associated with gastric and duodenal ulcers and has been implicated in cases of gastric carcinoma.
- *Campylobacter* and *Helicobacter* are microaerophilic, requiring oxygen at concentrations less than that found in ambient air.

LEARNING ASSESSMENT QUESTIONS

1. A gram-negative bacillus isolated from a stool specimen produces clear colonies on MacConkey agar and yellow colonies on thiosulfate citrate bile salt sucrose medium. The isolate is subcultured to a sheep blood agar plate with an O/129 disk. The isolate is sensitive to O/129 and is oxidase-positive. Which organism would you suspect?
 - a. *Vibrio parahaemolyticus*
 - b. *Vibrio cholerae*
 - c. *Plesiomonas*
 - d. *Aeromonas*

2. A patient with hemochromatosis developed septicemia after ingesting contaminated shellfish. What is the mortality rate after infection with this organism?
 - a. 20% to 30%
 - b. 30% to 40%
 - c. 40% to 50%
 - d. Above 50%
3. Which of the following genera is typically microaerophilic?
 - a. *Helicobacter*
 - b. *Aeromonas*
 - c. *Plesiomonas*
 - d. *Vibrio*
4. *Campylobacter jejuni* is most noted for causing which condition?
 - a. Wounds
 - b. Septicemia
 - c. Gastric ulcers
 - d. Gastroenteritis
5. Which of the following is a risk factor for acquiring *Vibrio alginolyticus* infection?
 - a. Farming
 - b. Hunting
 - c. Fishing or swimming in ocean water
 - d. Drinking unpasteurized milk
6. An oxidase-positive, indole-positive, β -hemolytic, gram-negative bacillus isolated from an adult stool culture is resistant to O/129, cannot grow in 6% NaCl broth, and is Voges-Proskauer positive. Which organism would you suspect?
 - a. *Aeromonas hydrophila*
 - b. *Aeromonas caviae*
 - c. *Plesiomonas shigelloides*
 - d. *Vibrio parahaemolyticus*
7. Darting motility is a characteristic of which organism?
 - a. *Aeromonas*
 - b. *Campylobacter*
 - c. *Vibrio cholerae*
 - d. *Arcobacter*
8. Which of the following tests is most helpful in differentiating *C. jejuni* from the other *Campylobacter* spp.?
 - a. Nitrate reduction
 - b. Urease activity
 - c. Hippurate hydrolysis
 - d. Susceptibility to nalidixic acid
9. When attempting to recover enteric *Campylobacter* spp., which specimen, media, and incubation conditions should be used?
10. Which nonculture methods are used to diagnose *Helicobacter pylori* infections?

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Nonfermenting and miscellaneous gram-negative bacilli

Yousif Barzani

CHAPTER OUTLINE

General characteristics of nonfermenters, 475

Clinical infections, 475

Biochemical characteristics and identification, 475

Clinically significant nonfermentative, gram-negative bacilli, 476

Pseudomonas fluorescent group, 481

Pseudomonas nonfluorescent group, 483

Acinetobacter, 484

Stenotrophomonas maltophilia, 485

Burkholderia, 486

Moraxella, *Oligella*, and *Psychrobacter*, 488

Less commonly encountered nonfermentative, gram-negative bacilli, 488

Alcaligenes and *Achromobacter*, 488

Brevundimonas, 489

CDC groups EO-3, EO-4, and *Paracoccus*, 490

Chromobacterium, 490

Comamonas and *Delftia*, 490

Weeksellaceae, 490

Methylobacterium and *Roseomonas*, 491

Ralstonia and *Cupriavidus*, 492

Shewanella, 492

Sphingomonas, 492

Bibliography, 494

OBJECTIVES

After reading and studying this chapter, you should be able to:

1. Describe the general characteristics of nonfermentative, gram-negative rods.
2. Compare the metabolic pathways used by nonfermentative organisms with those used by fermentative organisms.
3. Discuss the natural habitat of the nonfermentative, gram-negative bacilli.
4. Describe the types of infections that nonfermentative organisms cause and the patients who are at high risk for infections.
5. Characterize the most clinically significant nonfermentative gram-negative bacilli.
6. Discuss the incidence of infections caused by various nonfermenters in patients with cystic fibrosis.
7. Recognize the initial clues to nonfermentative organisms when isolated in clinical specimens.
8. Describe the typical biochemical reactions and key characteristic features of the most commonly encountered nonfermentative organisms.
9. Evaluate the typical antimicrobial susceptibility pattern of the most commonly encountered nonfermentative organisms.
10. Justify when a full identification and antimicrobial susceptibility test should be performed on a nonfermenting gram-negative bacillus.

KEY TERMS

Asaccharolytic

Eugonic oxidizer (EO)

Fermentative

Nonfermentative

Nonoxidizers

Oxidizers

Pseudomonas

Pyocyanin

Pyoverdine

Case in point

A 25-year-old female patient who received a bone marrow transplant as treatment for aplastic anemia presented with fever, chills, and malaise of about 2 days' duration. Two sets of blood cultures were drawn, 20 mL each. The patient was admitted, and because her fever persisted, another two sets of blood cultures were collected about 5 hours later. At this point, the patient was given a broad-spectrum antimicrobial agent, with the plan to give her antifungal agents if the fever persisted. About 12 hours after the first blood cultures had been drawn, both blood culture bottles in set one were positive for a gram-negative bacillus. Four hours later, the other two sets were also positive for a gram-negative bacillus. The next day, the clinician was informed that the gram-negative bacillus was an oxidase-positive, nonlactose fermenter. The laboratory

had recently instituted use of mass spectrometry for identification of gram-negative bacilli, and the final identification of the isolate was reported a few hours later, with an antimicrobial susceptibility test performed thereafter.

Issues to consider

After reading the patient's case history, consider:

- The risk factors in this patient for infection with a nonfermentative gram-negative bacillus
- The significance of the number of positive blood cultures and the time until the cultures were positive
- How you might rapidly identify this organism for better clinical care

Gram-negative bacilli can be divided into aerobic and obligate anaerobic bacilli. Aerobic gram-negative bacilli can be further divided into at least two large groups: those that ferment carbohydrates, called **fermentative** or *fermenters*, and those that do not ferment carbohydrates, called **nonfermentative** or *nonfermenters*. This chapter discusses miscellaneous nonfermentative organisms that are clinically significant because of the increasing numbers of patients who are immunocompromised. This group of patients often has multiple risk factors for infection with these normally uncommon pathogens. The groups of organisms discussed in this chapter include *Pseudomonas* spp., *Acinetobacter* spp., *Stenotrophomonas maltophilia* and *Burkholderia* spp., and other species that fail to metabolize carbohydrates.

General characteristics of nonfermenters

Nonfermenting gram-negative bacilli fail to acidify an oxidative-fermentative (OF) medium when it is overlaid with mineral oil or fail to acidify triple sugar iron agar (TSIA) butts. They prefer and grow much better in an aerobic environment; some do not grow in an anaerobic environment at all. Some group members oxidize carbohydrates to derive energy; they are referred to as **oxidizers**. Others do not break down carbohydrates at all; they are referred to as **nonoxidizers** or **asaccharolytic**. Additional characteristics can differentiate this group of nonfermenters from other gram-negative bacilli—motility, pigmentation, and their ability or lack of ability to grow on selective gram-negative media such as MacConkey (MAC) agar. Most nonfermentative, gram-negative bacilli are oxidase positive, a feature that differentiates them from the members of the order Enterobacterales (*Plesiomonas* sp. is the only oxidase-positive member of the order Enterobacterales). A variety of approaches to the phenotypic identification of aerobic gram-negative bacilli are available.

In general, nonfermentative, gram-negative bacilli or coccobacilli are ubiquitous and found in most environments, typically in soil and water, on plants and decaying vegetation, and in many foodstuffs. They prefer moist environments, and in hospitals, they can be isolated from nebulizers, dialysate fluids, saline, catheters, and other devices. Nonfermenters can withstand treatment with chlorhexidine and quaternary ammonium compounds. They are rarely, if ever, part of the normal host microbiota but can easily colonize hospitalized

patients, especially those who are immunocompromised. Many of the nonfermentative, gram-negative bacilli tend to be resistant to multiple classes of antimicrobial agents.

Clinical infections

Nonfermenters account for about 15% of all gram-negative bacilli isolated from clinical specimens. Although there are differences among the clinical diseases caused by each species, some common disease manifestations and risk factors can be found. Nonfermenters are responsible for a number of serious infections, including septicemia, meningitis, osteomyelitis, and wound infections, usually following surgery or trauma. Common risk factors for development of these infections are listed in [Box 21.1](#). Usually, the individual who contracts an infection with one of these organisms is hospitalized or has been discharged recently from the hospital.

Biochemical characteristics and identification

As noted, all nonfermenters fail to ferment carbohydrates and thus will not yield acidic reactions in the anaerobic portion of media, such as TSIA or Kligler iron agar (KIA). A fermenter (e.g., *Escherichia coli*) typically produces an acid (yellow) butt with an acid or alkaline (red) slant on TSIA or KIA within 18 hours of incubation on either of these media. A nonfermenter (an oxidizer or nonoxidizer) produces no change in the butt and slant or may produce an alkaline (red) slant. The principles of the TSIA reactions are explained in [Chapter 9](#). In addition to these types of organisms, some “true” fermenters are fastidious (e.g., *Pasturella multocida*) and do not easily acidify the butt or slant of a TSIA like other fermenters, but they do show reactions if more enriched media are used. Some characteristic clues can indicate the presence of a nonfermenter in the clinical laboratory:

- Oxidase-positive reaction, although reaction can be weak and variable

BOX 21.1 Risk factors for diseases caused by nonfermentative, gram-negative bacilli

Immunosuppression

Diabetes mellitus
Cancer
Steroids
Transplantation

Trauma

Gunshot, knife wounds, punctures
Surgery
Burns

Foreign body implantation

Catheters, urinary or bloodstream
Prosthetic devices—joints, valves
Corneal implants or contact lenses

Infused fluids

Dialysate
Saline irrigations

- Nonreactivity in 24 hours in commercial multitest kit systems used primarily for the identification of Enterobacterales
- No acid production in the slant or butt of TSIA or KIA
- Resistance to a variety of classes of antimicrobial agents, such as aminoglycosides, third-generation cephalosporins, penicillins, and fluoroquinolones

Many classification systems have been devised for grouping the nonfermenters. One system uses the reactions of three common tests: (1) growth on MAC agar, (2) oxidase reaction, and (3) the glucose OF test. The eight possible combinations of results are then used to group the nonfermenters, as shown in Fig. 21.1. Included in this figure are some isolates of nonfermentative, gram-negative bacilli not always considered with the more typical nonfermenters. Organisms such as *Brucella* spp. and *Bordetella* spp. are biochemically similar and are discussed in Chapter 18. For the identification of nonfermentative, gram-negative bacilli, conventional tube biochemical testing, multitest kit systems, automated systems, molecular biology methods involving deoxyribonucleic acid (DNA), or more recently, matrix-assisted laser desorption/ionization–time-of-flight (MALDI-TOF) mass spectrometry can be used.

Four groups of nonfermenters make up most isolates routinely seen in clinical laboratories: *Pseudomonas aeruginosa*, *Acinetobacter* spp., *Burkholderia* spp., and *Stenotrophomonas maltophilia*. Any of the aforementioned systems perform adequately to identify these four groups. For the remainder of the nonfermenters, for which there remains great variability in the accuracy of identification depending on the system used, decisions must be made about whether a full identification is needed based on the clinical situation and relevance of the isolate. As a result of nucleic acid studies, the nonfermentative, gram-negative bacilli continually undergo taxonomic changes. Table 21.1 lists the present taxonomy of many nonfermenters. Box 21.2 summarizes characteristics common to some gram-negative nonfermenters.

The decision to identify many of the nonfermenters is based on the site from which they have been isolated; that is, whether they have been recovered from a normally sterile site in pure culture or from a nonsterile site in which three or four other bacterial species are also present. In the former case, it may be decided that definitive identification and susceptibility testing are required. In the nonsterile site, genus identification might be appropriate, which can be achieved through use of a few biochemical tests (e.g., oxidase, growth on MAC agar, glucose utilization, indole, and motility). Alternatively, identification as a nonfermenter, not *P. aeruginosa*, *Acinetobacter* spp., *Burkholderia* spp., or *S. maltophilia*, may be all that is needed, especially when cultures are mixed with many other bacterial species.

Definitive identification of every nonfermenter is time-consuming and can be costly. Fig. 21.2 illustrates typical biochemical and morphologic characteristics for many of the more common nonfermentative, gram-negative bacilli. If definitive identification is considered necessary for epidemiologic purposes or for academic reasons, such as for publication of an unusual case report involving the organism, clinical laboratory scientists should consider sending the isolate to a reference laboratory that is better equipped to achieve these identifications in a cost-effective manner. Today, many

reference laboratories use nucleic acid sequencing or mass spectrometry for identification rather than relying on biochemical or phenotypic identification for obtaining a definitive answer.

MALDI-TOF mass spectrometry identifies bacteria and fungi from colonies based on analysis of the unique spectra of their peptides when separated in a mass spectrometer; see Chapter 11. The identification can be made within minutes with a high level of accuracy and reproducibility compared with the gold standard of molecular sequencing. The mean time to identification of one isolate is 6 minutes, with a cost estimated at 20% to 30% lower than the conventional procedures that use phenotypic methods. In a study that compared biochemical methods with MALDI-TOF mass spectrometry using a 16S ribosomal ribonucleic acid (rRNA) gene sequencing method as the reference method for comparison, of 173 species-level identified strains from 187 clinical samples, 75% were identified correctly by API 20NE (bioMérieux, Durham, NC) strip, 83% by the VITEK 2 system (bioMérieux), and 89% by MALDI-TOF mass spectrometry. When the easily identified *S. maltophilia* strains were excluded, genus-level identification was 93% with MALDI-TOF mass spectrometry versus 76% with the API 20NE system and 81% with the VITEK 2 system.

For clinically significant isolates of *P. aeruginosa*, *S. maltophilia*, *Acinetobacter* spp., and *Burkholderia cepacia*, the Clinical and Laboratory Standards Institute (CLSI) recommends that a broth microdilution or Kirby-Bauer disk diffusion assay be performed. For other species, broth microdilution, not disk diffusion, is recommended if antimicrobial susceptibility testing is done at all.

Case check 21.1

When trying to determine the relevance of a particular isolate in a patient's culture, a physician often uses a combination of factors, including the risk factors that a patient may have for a variety of microbes. The relevance of isolation of nonfermentative bacilli from the sputum of an immunocompetent, healthy individual with a cough might differ from that in patients who are taking heavy doses of steroids because they have just received a lung transplant and they have cystic fibrosis (CF). Laboratories should consider contacting physicians when unusual isolates are cultured from clinical specimens to determine whether the workup should be more extensive in patients who have underlying risk factors. The patient in the Case in Point was a bone marrow transplant recipient and would fall into the at-risk group for development of infections with nonfermenters.

Clinically significant nonfermentative, gram-negative bacilli

The genus *Pseudomonas* accounts for the largest percentage of all nonfermenters isolated from clinical specimens. Characteristics common to most of the **pseudomonads** and several *Pseudomonas*-like organisms, include:

- Gram-negative bacillus or coccobacillus
- Strictly aerobic metabolism
- Motile, usually with polar flagellum or polar tuft of flagella

Table 21.1 Taxonomic changes for some gram-negative nonfermenters

New name	Old name
<i>Achromobacter xylosoxidans</i>	<i>Achromobacter xylosoxidans</i> var. <i>xylosoxidans</i>
<i>Achromobacter denitrificans</i>	<i>Achromobacter xylosoxidans</i> var. <i>denitrificans</i>
<i>Bergeyella zoohelcum</i>	<i>Weeksella zoohelcum</i>
<i>Brevundimonas diminuta</i>	<i>Pseudomonas diminuta</i>
<i>Burkholderia mallei</i>	<i>Pseudomonas mallei</i>
<i>Chryseobacterium gleum</i>	<i>Flavobacterium gleum</i>
<i>Chryseobacterium indologenes</i>	<i>Flavobacterium indologenes</i>
<i>Cupriavidus pauculus</i>	<i>Ralstonia pauculus</i>
<i>Cupriavidus gilardi</i>	<i>Ralstonia gilardi</i>
<i>Delftia acidovorans</i>	<i>Comomonas acidovorans</i>
<i>Elizabethkingia meningoseptica</i>	<i>Chryseobacterium meningosepticum</i>
<i>Empedobacter brevis</i>	<i>Flavobacterium brevis</i>
<i>Methylobacterium mesophilicum</i>	<i>Pseudomonas mesophilica</i>
<i>Myroides odoratus</i>	<i>Flavobacterium odoratum</i>
<i>Neisseria animaloris</i>	CDC group EF-4a
<i>Neisseria zoodegmatidis</i>	CDC group EF-4b
<i>Ochrobactrum anthropi</i>	<i>Achromobacter</i> biovar 1, 2, or Vd-1
<i>Pandoraea</i> spp.	CDC WO-2
<i>Paracoccus yeei</i>	CDC group EO-2
<i>Pseudomonas luteola</i>	<i>Chryseomonas luteola</i>
<i>Psychrobacter phenylpyruvicus</i>	<i>Moraxella phenylpyruvica</i>
<i>Ralstonia mannitolilytica</i>	<i>Ralstonia pickettii</i> biovar 3/'thomasi'
<i>Ralstonia pickettii</i>	<i>Pseudomonas pickettii</i>
<i>Rhizobium radiobacter</i>	<i>Agrobacterium radiobacter</i>
<i>Sphingobacterium multivorum</i>	<i>Flavobacterium multivorum</i>
<i>Sphingobacterium mizutaii</i>	<i>Flavobacterium mizutaii</i>
<i>Sphingobacterium spiritivorum</i>	<i>Flavobacterium spiritivorum</i>
<i>Sphingomonas paucimobilis</i>	<i>Pseudomonas paucimobilis</i> and CDC group IIk-1

CDC, Centers for Disease Control and Prevention.

- Oxidase positive (except *P. luteolus* and *P. oryzihabitans*)
- Catalase positive
- Usually grows on MAC agar
- Usually an oxidizer of carbohydrates, but some species are asaccharolytic

Many manual and automated commercial systems are available for the identification of nonfermenters. The Crystal enteric nonfermenter system (Becton Dickinson, Franklin Lakes, NJ) includes a database of 24 taxa of

BOX 21.2 Characteristics common to groups of nonfermenters

Pigmentation

Yellow

Chryseobacterium and *Elizabethkingia* spp. (weak fermenters)
Sphingomonas paucimobilis
Pseudomonas (Chryseomonas) luteola
Pseudomonas oryzihabitans
Sphingobacterium spp.
Pseudomonas stutzeri (light yellow) and wrinkled colonies

Pink

Methylobacterium spp.
Roseomonas spp.

Purple (MacConkey agar)

Acinetobacter sp.

Blue-green

Pseudomonas aeruginosa

Violet

Chromobacterium violaceum (fermenter)

Lavender to lavender-green (blood agar)

Stenotrophomonas maltophilia

Tan (occasionally)

Pseudomonas stutzeri
Shewanella putrefaciens

Wrinkled colonies

P. stutzeri
Pseudomonas oryzihabitans
Burkholderia pseudomallei

Odor

Sweet

Alcaligenes faecalis
Myroides odoratus
P. aeruginosa (grapes)

Popcorn

EO-4
Neisseria zoodegmatidis

Nonmotile

Acinetobacter spp.
Moraxella spp.
Chryseobacterium spp. and *Elizabethkingia* spp. (weak fermenters)
Sphingobacterium spp. (may "glide")
Oligella spp. (non-*O. ureolytica*)

Oxidase negative

Acinetobacter spp.
S. maltophilia
Pseudomonas luteola and *P. oryzihabitans*
Burkholderia cepacia

H₂S positive

S. putrefaciens

Courtesy Anne Morrissey (Cleveland, OH).

		MOTILE, STRONGLY SACCHAROLYTIC NONFERMENTERS										MOTILE, WEAK, OR NONSACCHAROLYTIC NONFERMENTERS											
		<i>Pseudomonas aeruginosa</i>	<i>Pseudomonas fluorescens/putida</i>	<i>Burkholderia cepacia</i>	<i>Achromobacter xylosoxidans</i>	<i>Stenotrophomonas maltophilia</i>	<i>Burkholderia pseudomallei</i>	<i>Pseudomonas stutzeri</i>	<i>Sphingomonas paucimobilis</i>	<i>Pseudomonas mendocina</i>	<i>Ralstonia pickettii</i>	<i>Delftia acidovorans</i>	<i>Pseudomonas pseudoalcaligenes</i>	<i>Alcaligenes faecalis</i>	<i>Achromobacter denitricans</i>	<i>Oligella ureolytica</i>	<i>Bordetella bronchiseptica</i>	<i>Pseudomonas alcaligenes</i>	<i>Brevundimonas diminuta</i>	<i>Brevundimonas vesicularis</i>	<i>Comamonas testosteroni</i>	<i>Shewanella putrefaciens</i>	
Oxidase		+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Pyocyanin		+/-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Fluorescein		+	-/+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Glucose		+	+	+	+/-	+/-	+	+	+/-	+	+	-	-/+	-	-	-	-	-	-	+	-	-	
Xylose		+	+	+/-	+	-	+	-/+	+/-	+	+	-	-	-	-	-	-	-	-	-	-	-	
Mannitol		+/-	+/-	+/-	-	-	+	-/+	-	-	-	+/-	-	-	-	-	-	-	-	-	-	-	
Lactose		-	-	+/-	-	-	+	+/-	+/-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Maltose		-	-	+/-	-	+	+	+/-	+/-	-	-	-	-/+	-	-	-	-	-	-	+	-	-	
42° C		+	-	+/-	+	+/-	+	+	-	+	+/-	-	+	+/-	-/+	-	+	+/-	-/+	+/-	+/-	+/-	
Esculin		-	-	-/+	-	+	+/-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-	
Urea		+	-/+	+/-	-	+/-	-/+	-/+	-/+	+/-	+	-	-	-/+	-	+	+	-/+	+/-	-	-	+/-	
DNase		-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	+	+	-	+	
ONPG		-	-	+/-	-	+	-	-	+/-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Indole		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Motility		+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	
Flagella		1	>1	>1	P	>1	>1	1	1	1	1	>1	1	P	P	P	P	1	1	1	>1	1	
H ₂ S		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	
N ₂ gas		+/-	-	-	-	-	+	+	-	+	-/+	-	-	+	+/-	+	-	-	-	-	-	-	
Pigment		B,F,G	F	Y	-	Y	-	B,Y	Y	-	-	-	-	-	-	-	-	-	-	B,Y	-	B	
Growth on MacConkey agar		+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-/+	+/-	+	

Fig. 21.2 Biochemical and morphologic characteristics of selected nonfermentative gram-negative bacilli. +, Most strains positive; —, most strains negative; B, brown; F, fluorescein; G, green; ONPG, o-nitrophenyl-β-D-galactopyranoside; Y, yellow. (Data from Ohio State University Hospital, Columbus, OH.)

	NONMOTILE, PIGMENTED, INDOLE-POSITIVE NONFERMENTERS				NONMOTILE COCCOBACILLI	
					Oxidase negative	
	<i>Elizabethkingia meningosepticum</i>	<i>Myroides odoratus</i>	<i>Bergeyella zoohelcum</i>	<i>Weeksella virosa</i>	<i>Moraxella</i> sp.	<i>Acinetobacter lwoffii</i>
						<i>Acinetobacter baumannii</i>
Oxidase	+	+	+	+	+	-
Pyocyanin	-	-	-	-	-	-
Fluorescein	-	-	-	-	-	-
Glucose	+/-	-	-	-	-	+
Xylose	-	-	-	-	-	+
Mannitol	-/+	-	-	-	-	-
Lactose	-	-	-	-	-	+
Maltose	-/+	-	-	-	+/-	-
42° C	+/-	-	-	-/+	-/+	+
Esculin	+	-	-	-	-	-
Urea	-/+	+	+	-/+	-	-/+
DNase	+	+	+	+	-	-/+
ONPG	+/-	-	-	-	-	-
Indole	+	-	-/+	+	-	-
Motility	-	-	-	-	-	-
Flagella	-	-	-	-	-	-
H ₂ S	-	-	-	-	-	-
N ₂ gas	-	-/+	-	-	-	-
Pigment	Y	Y	B	B	-	-
Growth on MacConkey agar	+	+	-	-	+/-	+

Fig. 21.2 cont'd

Flagella

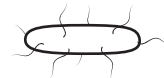
1, Polar monotrichous



>1, Polar tuft (>1 flagellum)



P, Peritrichous



nonfermenters, consisting of 10 different genera. There are an additional 20 taxa of miscellaneous gram-negative bacilli included in the database. The ability to use one manual system to identify enterics and nonfermenters may be attractive to a smaller laboratory, even if the database

is limited, as it is in this system. The API 20NE is specifically for the identification of nonfermenters, and the API 20E is for enterics. The API 20NE performs well for most commonly isolated nonfermenters and is used in many clinical laboratories. The Remel RAPID NF Plus System

(Thermo Fisher Scientific, Waltham, MA) provides identification by analysis of preformed enzymes; 70 medically important nonfermenters, including members of the *B. cepacia* complex, can be identified in 4 hours. The Biolog microbial ID system (Biolog, Hayward, CA) is a microbial identification system that can be used manually or in fully automated format; the database contains over 2500 species of bacteria and yeasts, including most of the clinically relevant nonfermenters. The VITEK 2 and MicroScan systems (Beckman Coulter, Brea, CA) provide automated approaches to the identification of a variety of bacteria and yeasts that include many of the clinically relevant nonfermenters.

Today, MALDI-TOF mass spectrometry and nucleic acid sequencing are becoming more popular because of their ability to identify rapidly and accurately many more nonfermenters than can be done with phenotypic methods. The two instruments for performing MALDI-TOF mass spectrometry, the MALDI Biotyper (Bruker, Billerica, MA) and the VITEK MS, were compared for the identification of 200 nonfermenting bacilli isolated from CF patients at the University of Iowa Health Care. Both provided rapid and reliable (>89.5%) results with combined species, complex, and genus identification. More than 14 common and uncommon genera were included in this study. The two MALDI-TOF mass spectrometry instruments were compared in a study of 296 strains of many bacteria, including 49 nonfermenters. They provided very good identification, at least to the genus level, compared with gold standard sequencing identification. Identification of nonfermenters by 16S rRNA sequencing was compared with that accomplished with the API 20NE and VITEK 2 ID-GNB cards. About 92% of the isolates could be assigned to a species and 8% to a genus with sequencing compared with 54% to a species and 7% to a genus with the API 20NE, and 53% and 1% with VITEK 2 identification; 15% and 43%, respectively, were not found in the API 20NE and VITEK 2 databases.

***Pseudomonas* fluorescent group**

Pseudomonas aeruginosa

Pseudomonas aeruginosa is the most commonly isolated species of the genus in clinical specimens. It is found in moist environments, including tap water, pools, hot tubs, catheters, and humidifiers in hospitals, and in plants and soil. It is an uncommon part of the normal bacterial microbiota and is isolated from less than 12% of normal stool specimens. It can cause mild illness in healthy people and severe infections in people with weak immune systems. It may, however, account for 5% to 15% of all healthcare-associated infections, especially pneumonia and bacteremia. *P. aeruginosa* is the leading cause of healthcare-associated respiratory tract infections. A large variety of clinical diseases have been documented as being caused by *P. aeruginosa*, including bacteremia, often presenting with ecthyma gangrenosum; wound infections; pulmonary disease, especially among individuals with CF; healthcare-associated urinary tract infections (UTIs); endocarditis; bone infections; eye infections, including keratitis, ulcers, and endophthalmitis; infections following burns or trauma; and in rare cases, central nervous system infections, including meningitis.

P. aeruginosa accounts for up to 6% of all bacteremias and as many as 75% of healthcare-associated bacteremias and is the third most common cause of gram-negative bacillary bacteremia, after *E. coli* and *Klebsiella pneumoniae*. It is a common cause of lung infections in people with CF. Poor prognostic factors associated with *P. aeruginosa* bacteremia include septic shock, granulocytopenia, inappropriate antimicrobial therapy, and the presence of septic metastatic lesions. When *P. aeruginosa* is isolated from a sterile body site, such as blood, pleural fluid, joint fluids or tissues, or cerebrospinal fluid (CSF), it almost always constitutes a true infection.

Because *P. aeruginosa* can colonize mucosal surfaces, such as the oropharynx, patients in the intensive care unit (ICU) who are mechanically ventilated may quickly become colonized with *P. aeruginosa*. Positive cultures might not always indicate infection, although *P. aeruginosa* is a common cause of ventilator-associated pneumonia, especially in the immunocompromised or otherwise severely ill patient. Fig. 21.3 illustrates the Gram-stained smear of a bronchial specimen containing *P. aeruginosa*. Other less serious conditions associated with *P. aeruginosa* infection are otitis externa, particularly in swimmers or divers; necrotizing rash, referred to as *Jacuzzi* or *hot tub syndrome*, that develops in users of these recreational facilities; and infections of the nail beds in people with artificial nails.

Virulence factors

A variety of factors contribute to the pathogenicity of *P. aeruginosa*, such as endotoxin (lipopolysaccharide [LPS]), motility, pili, capsule, flagella, phospholipases, a type III secretion system, and several exotoxins (Table 21.2). The most important exotoxin is exotoxin A; this exotoxin functions similarly to diphtheria toxin by blocking protein synthesis. In lower respiratory tract infections of patients with CF, most *P. aeruginosa* strains produce mucoid colonies caused by the overproduction of alginate, a polysaccharide polymer (Fig. 21.4). The production of mucoid colonies in strains isolated from patients with CF can be a helpful identifying characteristic. *P. aeruginosa* is also inherently resistant to a number of antimicrobial agents. Despite the vast array of virulence factors, *P. aeruginosa* is still considered an opportunistic pathogen.

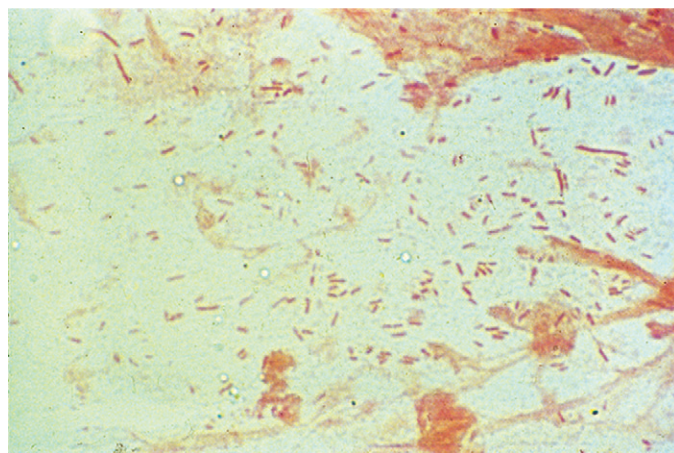


Fig. 21.3 Direct Gram-stained smear of bronchial specimen showing *Pseudomonas aeruginosa* (×1000).

Table 21.2 *Pseudomonas aeruginosa* virulence factors

Virulence factors	Function
Lipopolysaccharide	Antiphagocytic activity, cytotoxicity
Pili	Adhesion
Flagella	Motility, adhesion
Type III secretion system	Cytotoxic activity
Phospholipases	Cytotoxicity
Proteases	Cytotoxicity, proteolytic activity
Exotoxin A	Cytotoxicity
Capsule	Antiphagocytic activity
Deoxyribonuclease (DNase)	Breaks down DNA
Elastase	Cytotoxicity, proteolytic activity
Lecithinase	Cytotoxicity, proteolytic activity
Hemolysins	Cytotoxicity

LPS, Lipopolysaccharide.

Modified from Fauci, A. S., et al. (Eds.). (2008). *Harrison's principles of internal medicine* (17th ed.). New York: McGraw-Hill.

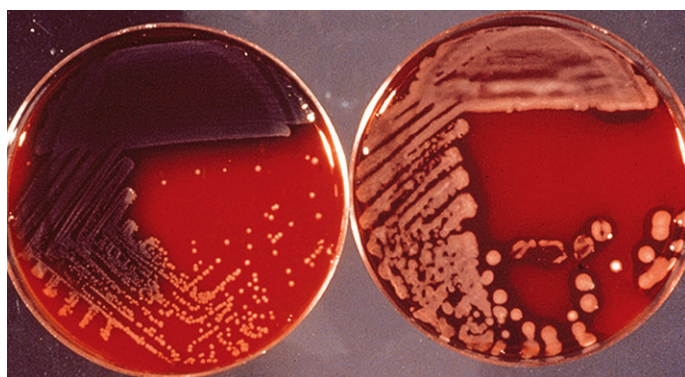


Fig. 21.4 *Pseudomonas aeruginosa* on sheep blood agar. Left, Nonmucoid colonies. Right, Mucoid colonies. Note discoloration of media, especially on the left.

Case check 21.2

When a nonfermentative bacillus is isolated from a clinical specimen in the laboratory, it could represent a pathogen, a saprophytic bacterium that has become part of the microbiota of the patient, or contamination of the specimen that occurred during specimen collection, transport, or processing in the laboratory. The goal of the laboratory is to give accurate information to the clinical team so that they can best evaluate and interpret the results of the culture. More significance is often given to isolates of nonfermenters when they are isolated from normally sterile sites or isolated in large quantities from many different samples. In the Case in Point, *P. aeruginosa* was isolated from four blood cultures and grew relatively quickly, indicating that this is likely the pathogen responsible for the patient's symptoms.

Identifying characteristics

Members of the *Pseudomonas* fluorescent group, which includes *P. aeruginosa*, *P. fluorescens*, *P. putida*, *P. veronii*, *P. mosselii*, and *P. monteilii*, produce **pyoverdinin**, a yellow-green or yellow-brown pigment. Pyoverdinin is water soluble and fluoresces under short-wavelength ultraviolet light. Most strains of *P. aeruginosa* will also produce the blue, water-soluble

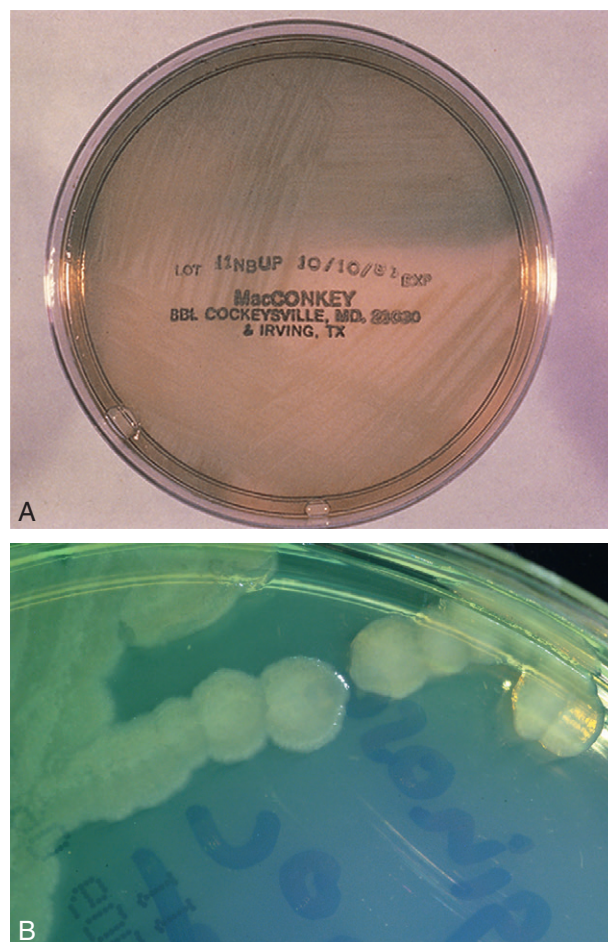


Fig. 21.5 *Pseudomonas aeruginosa* on MacConkey agar (A) and Mueller-Hinton agar (B). Note the blue-green pigment.

pigment **pyocyanin**. Pyocyanin combining with pyoverdinin produces the green color characteristic of *P. aeruginosa* colonies (Fig. 21.5). No other nonfermentative, gram-negative bacillus produces pyocyanin, so its presence can be used to specifically identify *P. aeruginosa*. If needed, pyocyanin typing can be used to epidemiologically link strains of *P. aeruginosa*. About 4% of clinical strains of *P. aeruginosa*, however, do not produce pyocyanin. Other water-soluble pigments, e.g., pyorubin (red) and pyomelanin (brown or black), are occasionally produced by strains of *P. aeruginosa*.

Most isolates of *P. aeruginosa* are β -hemolytic on sheep blood agar (SBA) and will produce flat, spreading colonies with a characteristic metallic sheen. Many strains also produce a fruity, grapelike odor caused by the presence of 2-aminoacetophenone. Other key characteristics of *P. aeruginosa* include denitrification of nitrates and nitrites, positive for arginine dihydrolase (ADH), growth at 42°C, citrate positivity, and acetamide utilization. Cetrimide agar is a selective and differential medium for the identification of *P. aeruginosa*. Cetrimide acts as a detergent and inhibits most bacteria; the medium also enhances the production of the two pigments produced by *P. aeruginosa*.

Treatment

P. aeruginosa is innately resistant to many antimicrobial agents, including penicillin, ampicillin, first- and second-generation cephalosporins, trimethoprim-sulfamethoxazole (SXT), chloramphenicol, and tetracyclines, along with other agents to which

most gram-negative bacilli are resistant. *P. aeruginosa* is usually susceptible to the aminoglycosides, semi-synthetic penicillins such as piperacillin and ticarcillin, third- and fourth-generation cephalosporins (ceftazidime and cefepime, respectively), carbapenems (except ertapenem), and the fluoroquinolones. Resistance to any of these agents may, however, develop while a patient is receiving therapy. The incidence of resistance is much higher in healthcare-associated strains of *P. aeruginosa*. Treatment of severe *P. aeruginosa* infections usually requires combination therapy, often with ceftazidime or cefepime, piperacillin, or a carbapenem (imipenem or meropenem) with an aminoglycoside (tobramycin or amikacin). Multidrug-resistant *P. aeruginosa* infections can be fatal in critically ill patients.

Pseudomonas fluorescens and *Pseudomonas putida*

Both *Pseudomonas fluorescens* and *P. putida* are of very low virulence, rarely causing clinical disease. They have been isolated from respiratory specimens, contaminated blood products, urine, cosmetics, hospital equipment, and fluids. *P. fluorescens* and *P. putida* have been documented, although rarely, as causes of UTIs, postsurgical abscesses, empyema, septic arthritis, and other wound infections. *P. putida* has been associated with catheter-related sepsis in patients with cancer; isolation of *P. fluorescens* from blood culture bottles in asymptomatic patients has been responsible for clusters of pseudobacteremia, probably related to contaminated catheters and catheter-related devices. Both species can grow at 4°C and have been linked to transfusion-associated septicemia.

P. fluorescens and *P. putida* produce pyoverdine, but neither produces pyocyanin or grows at 42°C, the key characteristics of *P. aeruginosa*. *P. fluorescens* and *P. putida* cannot reduce nitrate to nitrogen gas, but they can produce acid from xylose, characteristics that separate them from the other fluorescent group pseudomonads. Gelatin hydrolysis can be used to differentiate the two species from each other; *P. putida* is negative and *P. fluorescens* is positive. They are usually susceptible to the aminoglycosides, polymyxin, and piperacillin, but are resistant to carbenicillin and SXT.

Several more recently identified pseudomonads can be mistakenly identified as *P. fluorescens* by commercial phenotypic identification systems. These include *P. mosselii*, *P. veronii*, and *P. montelii*; sequence identification or MALDI-TOF mass spectrometry is needed for their specific identification. *P. mosselii* was first identified in 2002 during a study of strains of *P. fluorescens* and *P. putida*, when it was found that some isolates had low-level nucleic acid relatedness to other *Pseudomonas* strains. Colonies of *P. mosselii* are nonpigmented, nonhemolytic, and positive for oxidase, catalase, and ADH; optimal growth occurs at 30°C. A case of prosthetic valve endocarditis caused by *P. mosselii* was reported in the United Kingdom in 2009 that was successfully treated with piperacillin-tazobactam and ceftazidime. No surgery was needed to replace the valve, which was unusual compared with endocarditis caused by *P. aeruginosa*.

Case check 21.3

Laboratories often implement protocols when a susceptibility test would be performed on a nonfermenter. When *P. aeruginosa* is isolated from a sterile site such as a blood culture, most laboratories will provide this information as rapidly as possible so clinicians have the in vitro information they can use to develop a treatment regimen for their patient.

Pseudomonas nonfluorescent group

Pseudomonas stutzeri

Pseudomonas stutzeri, although a rare isolate and even rarer pathogen in the clinical laboratory, is usually easily recognizable because of its characteristic macroscopic appearance of wrinkled, leathery, adherent colonies that may produce a light-yellow or brown pigment (Fig. 21.6). Isolates are ADH negative and starch hydrolysis positive, the combination of which specifically distinguishes *P. stutzeri* from most other *Pseudomonas* spp. *P. stutzeri* is a soil denitrifier and can grow in an anaerobic environment in nitrate-containing media, producing nitrogen gas. This can differentiate the organism from other pseudomonads as well, although this is not a test commonly used in clinical laboratories.

In the immunocompromised host, *P. stutzeri* has been reported to be responsible for diseases that include septicemia, meningitis in people with human immunodeficiency virus infection, pneumonia (especially in patients with CF and those who are immunocompromised), endocarditis, postsurgical wound infections, septic arthritis, conjunctivitis, and UTIs. Isolates in vitro are usually susceptible to the aminoglycosides, SXT, ampicillin polymyxin, tetracyclines, fluoroquinolones, and third-generation cephalosporins (e.g., ceftazidime) but resistant to chloramphenicol and the first- and second-generation cephalosporins. Because of the adherent nature of colonies of *P. stutzeri*, identification of the organism and in vitro susceptibility testing can be unreliable.



Fig. 21.6 *Pseudomonas stutzeri* on sheep blood agar. Note the wrinkled appearance of the colonies.

Pseudomonas mendocina

Pseudomonas mendocina can be found in soil and water but is rarely isolated from human specimens; when it is, it is often considered a contaminant. There are rare cases of endocarditis described in the literature. *P. mendocina* produces nonwrinkled, flat colonies that may appear with a yellowish-brown pigment. Pigmentation seems to be a variable trait, but many exhibit a smooth buttery appearance. It is oxidase and ADH positive, like *P. aeruginosa*, but does not produce pyoverdine and is acetamide negative. *P. mendocina* is motile by means of a single polar flagellum, it oxidizes glucose and xylose, and is nonproteolytic and does not hydrolyze starch.

Pseudomonas pseudoalcaligenes and *Pseudomonas alcaligenes*

Pseudomonas pseudoalcaligenes and *Pseudomonas alcaligenes* are often considered contaminants when isolated from clinical specimens. They are oxidase positive and biochemically negative in many tests for which other *Pseudomonas* spp. test positive. They grow on MAC agar and are variable in the reduction of nitrates to nitrites or nitrogen gas. They are motile by means of a polar flagellum. *P. pseudoalcaligenes* is ADH positive and will weakly ferment fructose, two characteristics that differentiate it from *P. alcaligenes*. Commercial systems usually cannot discriminate the two species, and other methods of identification such as MALDI-TOF mass spectrometry or DNA sequencing are needed for definitive species differentiation. Few reports are available about the in vitro susceptibility of these species.

Pseudomonas luteola and *Pseudomonas oryzae*

The natural habitat of *Pseudomonas luteola* and *Pseudomonas oryzae* is unknown, although members of both genera have been isolated from soil and water. *P. oryzae* has been found in Japanese rice paddies and has been isolated from hospital drains and respiratory therapy equipment. These two pseudomonads are rarely isolated from humans but have been isolated from wounds, abscesses, blood cultures, peritoneal and chronic ambulatory peritoneal dialysis (CAPD) fluids, and other sources. They have also been implicated in cases of peritonitis and possibly meningitis, although often in association with each other or with other bacteria. *P. luteola* has been recovered as the only isolate from a case of prosthetic valve endocarditis and subdiaphragmatic abscess and from multiple brain abscesses in a child. *P. oryzae* has been isolated from the eye of one patient with postoperative endophthalmitis. There appears to be a higher risk for infection by these organisms in the presence of foreign materials (e.g., catheters), corticosteroid use, and immunocompromised states. A 9-year-old male patient was bitten by an octopus while swimming in the ocean and developed an infection with *P. oryzae*. The ulcerative wound healed once the lesion had been incised.

Both of these pseudomonads are gram-negative, nonfermentative, oxidase-negative bacilli. They are catalase positive and motile, oxidize glucose, grow on MAC agar, and often produce an intracellular nondiffusible yellow pigment. Both species typically produce wrinkled or rough colonies at 48 hours (Fig. 21.7). *P. luteola* can be differentiated from *P. oryzae* by positive *o*-nitrophenyl- β -D-galactopyranoside (ONPG) and esculin hydrolysis tests. *P. luteola* and *P. oryzae* are susceptible to aminoglycosides, third-generation

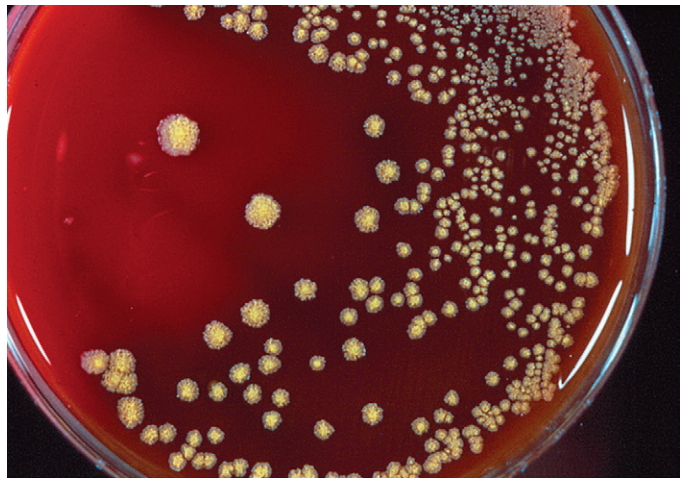


Fig. 21.7 *Pseudomonas oryzae* wrinkled yellow colonies on sheep blood agar plate at 48 hours.

cephalosporins, ureidopenicillins, and quinolones. *P. oryzae* isolates are resistant to first- and second-generation cephalosporins but are usually susceptible to penicillin. *P. luteola* is usually sensitive to all β -lactams.

Acinetobacter

The genus *Acinetobacter*, now a member of the family Moraxellaceae, consists of 25 DNA homology groups or genomospecies. Only about 12 species have been named; the two species most commonly seen in clinical specimens are *A. baumannii*, glucose-oxidizing, nonhemolytic, and *A. lwoffii*, glucose-non-oxidizer, nonhemolytic. Most hemolytic strains of *Acinetobacter* are *A. haemolyticus*. *Acinetobacter* spp. are ubiquitous in the environment in soil, water, and foodstuffs. In the hospital environment, they have been associated with ventilators, humidifiers, catheters, and other devices. About 25% of adults carry the organisms on their skin, and about 7% carry the organisms in their pharynx. If not already harboring *Acinetobacter* spp., hospitalized patients become easily colonized. As many as 45% of patients with a tracheostomy may be colonized. While severe infections by *A. baumannii* have been described, colonization is probably more common, and it is difficult to distinguish between the two conditions.

In the past, when *Acinetobacter* spp. were isolated from nonsterile sites such as urine and many different types of respiratory specimens, they were usually considered insignificant colonizers or contaminants. However, with increased isolates of *Acinetobacter* that demonstrate resistance to most antimicrobial agents, including the carbapenems, their clinical significance when isolated from respiratory or urine specimens in a hospitalized immunocompromised patient cannot be dismissed routinely. Ventilator-associated pneumonia and sepsis caused by *A. baumannii* have a high mortality rate.

Clinical infections

Acinetobacter spp. are opportunists, accounting for 1% to 3% of all healthcare-associated infections. They are second only to *P. aeruginosa* in frequency of isolation of all nonfermenters in the clinical microbiology laboratory. *Acinetobacter* infections primarily affect patients with weakened immune systems and coexisting diseases. These infections usually occur

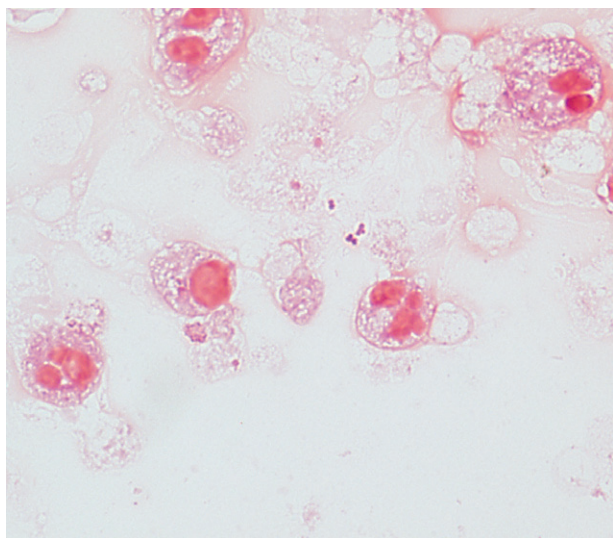


Fig. 21.8 *Acinetobacter baumannii* on Gram-stained smear. Note the coccoid nature of the cells as well as the pleomorphic forms ($\times 1000$).

in areas with a high fluid content such as the urinary tract and respiratory tract, and in peritoneal fluids. Diseases with which they have been associated (in particular *A. baumannii*) include UTIs; pneumonia, tracheobronchitis, or both; endocarditis, with up to a 22% mortality; septicemia; meningitis, often as a complication of intrathecal chemotherapy for cancer; and cellulitis, usually as a result of contaminated indwelling catheters, trauma, burns, or introduction of a foreign body. There are reports of eye infections caused by *A. baumannii*, including endophthalmitis, conjunctivitis, and corneal ulcerations.

Although *A. baumannii* has been rarely associated with skin and soft tissue infections in the past, it is now emerging as an important opportunistic multidrug-resistant (MDR) pathogen of skin and soft tissue infections. In 2004, the Centers for Disease Control and Prevention (CDC) reported an increased number of MDR *A. baumannii* bloodstream infections in U.S. military personnel returning from service in Iraq and Afghanistan who had suffered traumatic injuries. A fatal case of MDR *A. baumannii* soft tissue infection was reported in 2014 involving a 41-year-old morbidly obese man with history of alcoholic liver disease. *A. lwoffii* is much less virulent, and when isolated, it usually indicates contamination or colonization rather than infection.

Identifying characteristics

All *Acinetobacter* spp. are strictly aerobic, and they appear as gram-negative coccobacilli or even gram-negative cocci on Gram stain (Fig. 21.8). *Acinetobacter* organisms can resist decolonization and retain the crystal violet stain, leading to misidentification. They can appear as gram-positive cocci in smears made from blood culture bottles. *Acinetobacter* spp. are oxidase negative, catalase positive, and nonmotile. They have few growth requirements and thus are capable of growing on most laboratory media, including MAC agar. The purplish hue (from aerobic acidification of lactose) produced by some species on this medium may resemble that of a lactose-fermenting bacterium (Fig. 21.9). *A. baumannii* is saccharolytic, and *A. lwoffii* is asaccharolytic.

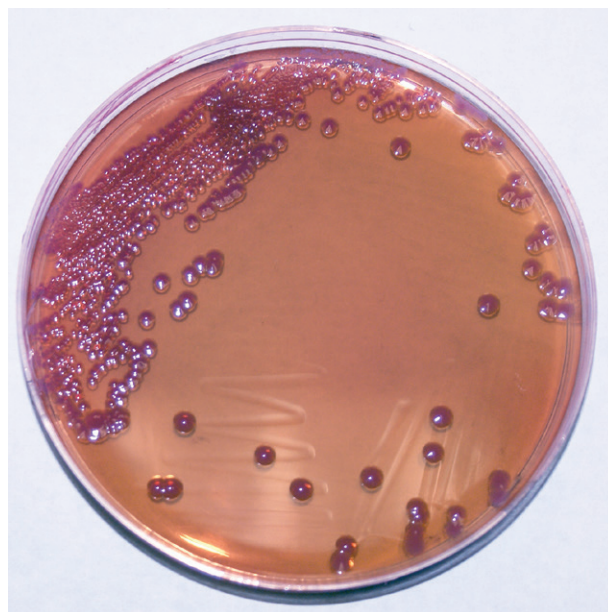


Fig. 21.9 *Acinetobacter baumannii* on MacConkey agar. This organism is saccharolytic, which may cause it to resemble a lactose-fermenting organism.

Isolates of *A. baumannii* are often resistant to many antimicrobials, including penicillins, first- and second-generation cephalosporins, and fluoroquinolones. *A. baumannii* demonstrates variable susceptibility to the aminoglycosides and β -lactam plus β -lactamase inhibitor combinations (e.g., ampicillin-sulbactam, piperacillin-tazobactam). Many strains exhibit resistance to carbapenems; carbapenemases have been reported throughout the United States. These isolates have been referred to as CRAB, or carbapenem-resistant *A. baumannii*. CRAB isolates are usually only susceptible to colistin and tigecycline. Isolation of patients in mobile isolation chamber units is often enforced when CRAB is isolated. *A. lwoffii* is susceptible to almost all antimicrobials, another characteristic distinguishing it from *A. baumannii*.

Stenotrophomonas maltophilia

Stenotrophomonas maltophilia is the third most common nonfermentative, gram-negative bacillus isolated in the clinical laboratory. Before 1983, it was a member of the genus *Pseudomonas*; it was later reclassified as a member of the plant pathogen genus *Xanthomonas*. After DNA homology and sequencing analysis, it was classified as a member of the genus *Stenotrophomonas*, where it remains today. Isolates are ubiquitous in the environment, being found in water, sewage, and plant materials; they are very common in the hospital environment, where they can be found contaminating blood-drawing equipment, disinfectants, transducers, and other equipment.

Clinical infections

When *S. maltophilia* is isolated from clinical specimens, it is initially regarded as a saprophyte or colonizer. Although not considered part of the normal human microbiota, *S. maltophilia* can quickly colonize the respiratory tract of hospitalized patients, in particular those exposed to antimicrobial agents to which *S. maltophilia* may be inherently resistant. These antimicrobials include cephalosporins, penicillins,

carbapenems, and aminoglycosides. There have been more reports of disease attributed to this organism, including endocarditis, especially in a setting of prior intravenous drug abuse or heart surgery; wound infections, including cellulitis and ecthyma gangrenosum; bacteremia; and, rarely, meningitis and UTIs. With rare exceptions, infections have occurred in a health care setting. *S. maltophilia* is rarely associated with lower respiratory tract infections, although it has been isolated from 6.4% to 10.2% of patients with CF.

In a retrospective study of 158 patients who had infections caused by nonfermenters other than *P. aeruginosa* and *A. baumannii*, 39% were caused by *S. maltophilia*, followed by *Achromobacter* spp. and non-*baumannii* *Acinetobacter* species. The most important risk factors in affected individuals were immunosuppression, hospitalization, especially in an ICU, and the presence of a central venous catheter. The mortality in patients with *S. maltophilia* infections was associated with inappropriate antimicrobial treatment because many were initially given a carbapenem. The association with inappropriate therapy and increased mortality was not seen with other non-fermenter infections.

Identifying characteristics

S. maltophilia is an oxidase-negative, nonfermentative, gram-negative bacillus. Colonies may appear bluish on MAC agar. In addition, it is positive for catalase, DNase, esculin and gelatin hydrolysis, and lysine decarboxylase. *S. maltophilia* is usually susceptible to SXT, so this is the drug of choice for most infections. Other agents to which it may demonstrate in vitro susceptibility include ticarcillin-clavulanate, the fluoroquinolone levofloxacin, and tetracyclines, including tigecycline. The CLSI recommends broth microdilution testing for *S. maltophilia*; in addition, an Epsilonometer test (Etest) or agar dilution can be performed.

Burkholderia

Burkholderia cepacia complex

Burkholderia cepacia complex contains at least 20 distinct genomic species (genomovars), and all species within the complex have been isolated from humans. *B. cepacia* complex contains plant pathogens that have arisen as opportunistic organisms, usually associated with pneumonia in patients with CF or chronic granulomatous disease (CGD). In addition, members of this complex have been reported to cause endocarditis (specifically in intravenous drug abusers), pneumonitis, UTIs, osteomyelitis, dermatitis, and other wound infections resulting from the use of contaminated water. They have been isolated from fluids used in the hospital (intravenous fluids and irrigation fluids), anesthetics, nebulizers, detergents, and disinfectants. Research supports the association of *B. cepacia* complex and increased severity of disease and death in patients with CF and CGD.

In the United States, the members of *B. cepacia* complex that account for most isolates recovered from clinical specimens are *B. cenocepacia* and *B. multivorans*. Other important isolates are *B. vietnamiensis*, *B. cepacia*, and *B. contaminans*. About 3% of the CF population is infected with *B. cepacia* complex, but rates up to 30% in some adult CF patients have been reported. Outside these populations, morbidity and mortality rates remain low, and consideration must be given



Fig. 21.10 *Burkholderia cepacia* on selective media. Left, Colonies of *B. cepacia* on oxidative-fermentative base, polymyxin B, bacitracin, and lactose (OFBBL) agar are yellow. Right, Colonies of *B. cepacia* on *Pseudomonas cepacia* agar are white, surrounded by a pink-red pigment.

to the possibility of contamination rather than infection when it is isolated in a non-CF or non-CGD individual.

The organisms grow well on most laboratory media but may lose viability on SBA in 3 to 4 days without transfers. Most members of the *B. cepacia* complex grow on MAC agar. Selective media containing antimicrobials to reduce the growth of *P. aeruginosa*, as well as other gram-negative bacilli, are available to increase the recovery of *B. cepacia* (Fig. 21.10). These include *Pseudomonas cepacia* oxidative-fermentative base, polymyxin B, bacitracin, and lactose (OFBL) agar, and *Burkholderia cepacia*-selective agar (BCSA). Studies suggest that BCSA is most effective in reducing overgrowth while maintaining good recovery of *B. cepacia*. Colonies of *B. cepacia* are nonwrinkled, and this trait may be used to differentiate isolates from *P. stutzeri*, which also produces a yellow pigment. *B. cepacia* does not fluoresce like *P. aeruginosa*, but it can produce a nonfluorescing yellow or green pigment that may diffuse into the media.

B. cepacia complex often produces a weak, slow, positive oxidase reaction. Almost all strains oxidize glucose, and many will oxidize maltose, lactose, and mannitol. Most strains are lysine decarboxylase positive, ONPG positive, ornithine decarboxylase negative, and fail to reduce nitrate to nitrite. Isolates are motile by means of polar tufts of flagella. Species identification among the genomovars of *B. cepacia* complex is difficult, and when a commercial system is used, confirmation should follow with supplemental biochemical, MALDI-TOF mass spectrometry, or molecular methods.

B. cepacia complex isolates are intrinsically resistant to aminoglycosides and polymyxins; in addition, many strains are resistant to several β -lactam antibiotics. Isolates may, however, be susceptible to chloramphenicol, ceftazidime, piperacillin, minocycline, some fluoroquinolones, and SXT. Susceptibility to the carbapenems is variable. Resistance can develop rapidly while a patient is being treated.

Burkholderia mallei

Burkholderia mallei causes glanders, a respiratory tract zoonosis primarily affecting livestock such as horses, mules, and donkeys. It is rare in humans but can produce severe local suppurative or acute pulmonary infections. The organism is considered by government agencies to be a potential bioterrorism agent; see Chapter 30. The only case of glanders in the United States in the past 50 years was caused by a laboratory accident in 2000; however, it is still endemic in parts of Africa, Asia, the Middle East, and Central and South America. India had remained free from glanders in livestock for more than 10 years when outbreaks occurred in 2006 to 2007 in which 164 horses were found positive in 8 Indian states. *B. mallei* is host adapted and does not grow in the environment.

B. mallei is a nonmotile, gram-negative coccobacillus that produces nonpigmented colonies in 2 days. Growth on MAC agar and oxidase production is variable; glucose is oxidized, nitrates are reduced to nitrites, and isolates are ADH positive. Like *B. cepacia* complex, isolates are resistant to polymyxins. If susceptibility tests are performed—and because of virulence should be performed only in approved laboratories—the CLSI recommends that a broth microdilution with *Brucella* broth and incubation at 35°C in ambient air for 16 to 20 hours be the method used. The drugs to consider testing for both *B. mallei* and *B. pseudomallei* should be ceftazidime, imipenem, doxycycline, and tetracycline.

Burkholderia pseudomallei

Burkholderia pseudomallei causes melioidosis, an aggressive, granulomatous, pulmonary disease caused by ingestion, inhalation, or inoculation of the organisms, with metastatic abscess formation in lungs and other viscera. Overwhelming septicemia can occur. Local infections, including orbital cellulitis, dacryocystitis, and draining abscesses, are sometimes seen, with pneumonia being the most common presentation. The incubation period may be prolonged, with reactivation occurring long after exposure.

The organisms are found in water and muddy soils in Southeast Asia (including Vietnam and Thailand), northern Australia, and Mexico. Those who have traveled to endemic areas are at risk for infection with *B. pseudomallei*. However, in 2021, four non-travel-related cases of *B. pseudomallei* infections were reported in several states including Georgia, Kansas, Texas, and Minnesota. None of the patients' families reported a history of traveling outside of the continental United States. The CDC investigation identified *B. pseudomallei* in an aromatherapy spray that was found in the home of the Georgia patient, and the genetic fingerprint of the bacteria in the bottle matched those of the bacteria identified in the four patients. Because the symptoms of melioidosis are varied and nonspecific, which may include pneumonia, abscess formation, and/or blood infections, the disease can initially be mistaken for other diseases such as tuberculosis, and proper treatment may be delayed.

This organism should be considered, especially when a nonfermentative wrinkled colony is isolated (Fig. 21.11) that demonstrates bipolar staining on Gram-stained smears. *P. stutzeri*, which can also appear as wrinkled colonies, does not use lactose, in contrast with *B. pseudomallei*. A selective medium, Ashdown medium, is supplemented with colistin; colonies on this agar are deep pink because of the absorption of neutral red in the medium. Colonies will also exhibit an earthy odor; however, work should be done in a biological

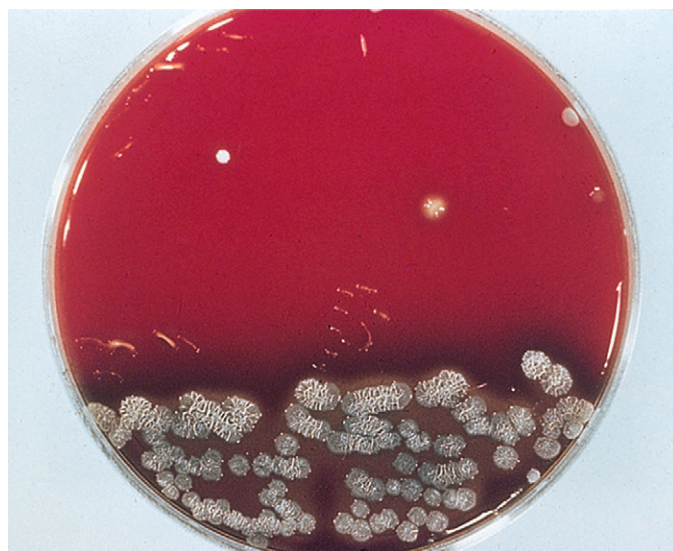


Fig. 21.11 *Burkholderia pseudomallei* on sheep blood agar.

safety cabinet when *B. pseudomallei* is suspected. As with all organisms, sniffing of plates is discouraged.

Identification of *B. pseudomallei* is generally accurate in many of the commercial manual nonfermenter systems. Identification involving automated algorithms (e.g., MALDI-TOF mass spectrometry, 16s, VITEK 2) has been shown unsuccessful and may misidentify *B. pseudomallei* as another bacterium. Use of multiplex polymerase chain reaction (PCR) assays for the identification of many of the *Burkholderia* spp., including *B. cepacia* complex and *B. pseudomallei*, has been shown to be of tremendous value to clinical microbiologists for rapid and accurate laboratory diagnosis. *B. pseudomallei* is considered a potential bioterrorism agent. If *B. pseudomallei* is isolated from someone who has never traveled to an endemic area, its occurrence should be reported to local or state public health departments.

Although isolates may be susceptible in vitro to many antimicrobial agents, including SXT, chloramphenicol, tetracycline, semi-synthetic penicillins, and ceftazidime, the clinical response to therapy is usually slow, and relapses are common. SXT and the fluoroquinolones seem to work well clinically. As with many of the nonfermenters, the CLSI does not recommend disk diffusion susceptibility testing.

Burkholderia gladioli

The plant pathogen, *Burkholderia gladioli* resembles *B. cepacia* complex. Isolates have been found in patients with CF and CGD. *B. gladioli* has been recovered from the blood and tissue of immunocompromised patients, particularly CF patients who have had a lung transplant. The 1-year (all-cause) mortality has been reported to be as high as 23%. A yellow pigment may be produced, especially after 48 to 72 hours of incubation. These organisms are motile by means of one or two polar flagella and are catalase and urease positive. *B. gladioli* grows on MAC agar, oxidizes glucose, is mannitol positive and decarboxylase negative, and is negative for oxidase, although some strains are weakly positive. Specific species identification of *B. gladioli* is difficult without the use of molecular tools for confirmation because isolates can be misidentified in commercial phenotypic systems as a member of the *B. cepacia*

complex. *B. gladioli* isolates are more susceptible to antimicrobials than *B. cepacia* and will be susceptible to aminoglycosides, carbapenems, ciprofloxacin, and SXT, but resistant to aztreonam and the cephalosporins, and 100% resistant to polymyxin B.

Moraxella*, *Oligella*, and *Psychrobacter

Moraxella and *Oligella*, along with *Acinetobacter* and *Psychrobacter*, are members of the family Moraxellaceae. *Moraxella* spp. are nonmotile, strongly oxidase-positive, gram-negative coccobacillary to bacillary organisms. They are, in general, biochemically inert with regard to carbohydrate oxidation (asaccharolytic). They are strictly aerobic and usually susceptible to penicillin, an unusual characteristic for a nonfermenter. These isolates are opportunists that reside on the mucous membranes of humans and lower animals and can be isolated from the respiratory tract, urinary tract, and eyes. However, they rarely cause disease in humans, with the exception of *M. catarrhalis*. Members of the genus *Moraxella* that are commonly encountered include *M. catarrhalis*, *M. nonliquefaciens*, *M. lacunata*, *M. osloensis*, *M. lincolnii*, and *M. atlantae*. *M. phenylpyruvica* has been reclassified as *Psychrobacter phenylpyruvicus*.

M. catarrhalis is the most frequent isolate in the genus *Moraxella* from clinical specimens, especially from respiratory and ear specimens. This species resembles the *Neisseria* by exhibiting gram-negative coccoid morphology, and at one time was named *Neisseria catarrhalis* and also *Branhamella catarrhalis*. *M. catarrhalis* is discussed in [Chapter 17](#) because of its phenotypic similarity to the *Neisseria*.

M. nonliquefaciens is the second most commonly isolated member of the genus. It often resides as normal biota in the respiratory tract and rarely causes disease in humans. Rare cases of bacteremia, keratitis, endocarditis, and endophthalmitis caused by *M. nonliquefaciens* have been reported. The organism does not usually grow on MAC agar. *M. osloensis* is similar morphologically and biochemically to *M. nonliquefaciens*; it is found as normal biota in the genitourinary tract. *M. lacunata* is a common conjunctival isolate.

Members of the genus *Oligella* include *O. urethralis* and *O. ureolytica*. *Oligella* are small, paired, gram-negative bacilli or coccoid organisms, usually isolated from the urinary tract. These organisms usually do not grow on MAC agar, are non-oxidative, phenylalanine deaminase (PDA) positive, oxidase positive, and nitrate and nitrite positive with gas formation. Most strains of *O. ureolytica* are motile by means of peritrichous flagella—hence, the relationship to *Alcaligenes* and *Taylorella*. The PDA positivity helps differentiate them from *Alcaligenes* spp. *O. urethralis* is nonmotile. *O. ureolytica* was reported to cause bacteremia in a patient with acquired immunodeficiency syndrome and in an 18-month-old child with pneumonia. *O. urethralis* is a commensal of the genitourinary tract, but it has been rarely associated with urosepsis, and there was a report of a case of infectious arthritis that was mistaken for gonococcal arthritis. *Oligella* spp. are usually susceptible to most antimicrobials, including penicillin, although resistance has been detected in some strains, and susceptibility testing should be performed if this is deemed clinically significant.

The genus *Psychrobacter* contains about 50 species. They have been isolated from fish, processed meat, and poultry. *Psychrobacter sanguinis* and *Psychrobacter phenylpyruvicus* are the species most frequently recovered from humans. Clinically, they have been isolated from the eye of a newborn, who had acquired the infection nosocomially via a

water source, and from the blood and CSF of a 2-day-old newborn. They have been isolated from urine, blood, CSF, and the genitourinary tract.

Psychrobacter spp. are nonmotile, oxidase-positive, oxidative diplococci. Isolates grow well at 5° to 25°C but rarely at 35°C. They are nitrate positive and can grow on modified Thayer-Martin medium. An odor of roses (resembling phenylethyl alcohol) has been reported. Tween 80 added to the medium stimulates growth. Isolates resemble *Moraxella* but are usually not penicillin susceptible. They are susceptible to most other antimicrobial agents.

Less commonly encountered nonfermentative, gram-negative bacilli

Alcaligenes* and *Achromobacter

The family Alcaligenaceae includes the clinically relevant genera *Alcaligenes*, *Achromobacter*, *Bordetella*, *Advenella*, and *Kertesia*. Isolates of both *Alcaligenes* and *Achromobacter* are found in water (e.g., swimming pools, tap water, dialysis fluids) and are resistant to disinfectants, such as chlorhexidine and quaternary ammonium compounds. They are isolated in specimens from hospitalized patients such as urine, feces, sputum, and wound specimens. The asaccharolytic members of this group are less likely isolated from clinical specimens than the saccharolytic species. Among the asaccharolytic members, *A. faecalis* is the species usually seen in clinical specimens, and it has been isolated from the blood of patients with and without septicemia. It has also been linked to eye infections, pancreatic abscesses, and other infections.

Achromobacter spp. can be divided into asaccharolytic species (*Achromobacter piechaudii* and *Achromobacter denitrificans*) and saccharolytic species (*Achromobacter xylosoxidans* and the unnamed *Achromobacter* groups B, E, and F). *A. piechaudii* has been isolated from the ear of a diabetic patient. Gram-stained smears of the exudate were positive for gram-negative bacilli and gram-positive cocci. Both coagulase-negative staphylococci and *A. piechaudii* were repeatedly isolated. The patient recovered once the diabetes was stabilized.

A. xylosoxidans is the most commonly isolated member of the genus *Achromobacter*. A 10-year analysis of 54 cases of *A. xylosoxidans* bacteremia demonstrated that it is a cause of nosocomial infection associated with the use of intravenous catheters; patients who are of advanced age and experiencing neutropenia are most susceptible to serious and sometime fatal (15%) illness. In addition, it has been associated with cases of otitis media, meningitis, pneumonia, surgical wound infections, UTIs, peritonitis, and bacteremia. It is a frequent colonizer in patients with CF. The genus *Advenella* contains one species, *A. incenata*, described in 2005. Although their significance is unclear, isolates have been recovered from sputum, wounds, and blood.

Alcaligenes faecalis and *Achromobacter* spp. possess peritrichous flagella ([Fig. 21.12](#)) and are obligately aerobic gram-negative bacilli. These organisms usually grow well on most laboratory media, including MAC agar. On SBA, most species are nonpigmented ([Fig. 21.13](#)). Some strains may produce a fruity odor and cause a green discoloration on SBA. In OF media, most isolates are nonoxidative and produce a deep blue color at the top, except for *A. xylosoxidans*, which produces an acid reaction with glucose and xylose (hence the

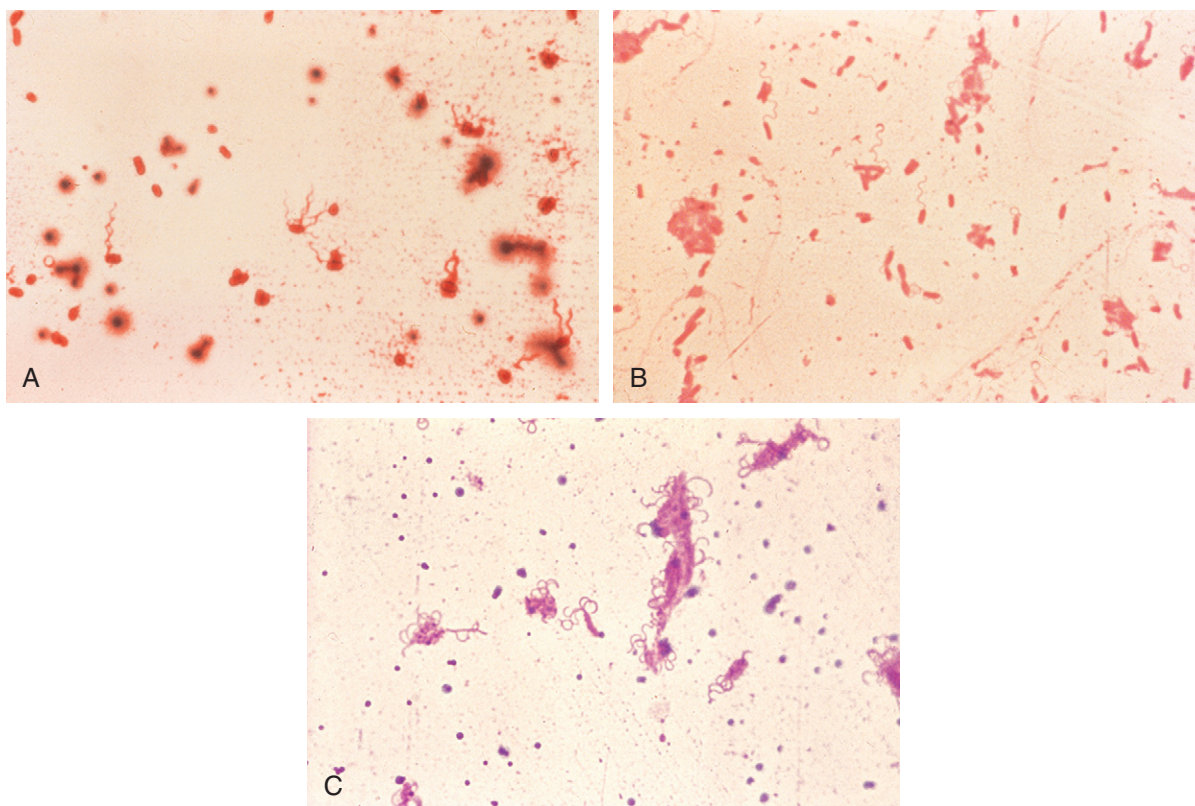


Fig. 21.12 Flagella stains of gram-negative bacilli. **A**, Peritrichous flagella (e.g., *Alcaligenes faecalis* and *Achromobacter* spp.) ($\times 1000$). **B**, Polar, monotrichous (e.g., *Pseudomonas aeruginosa*) ($\times 1000$). **C**, Polar, multitrichous (e.g., *Comamonas* spp.) ($\times 1000$).

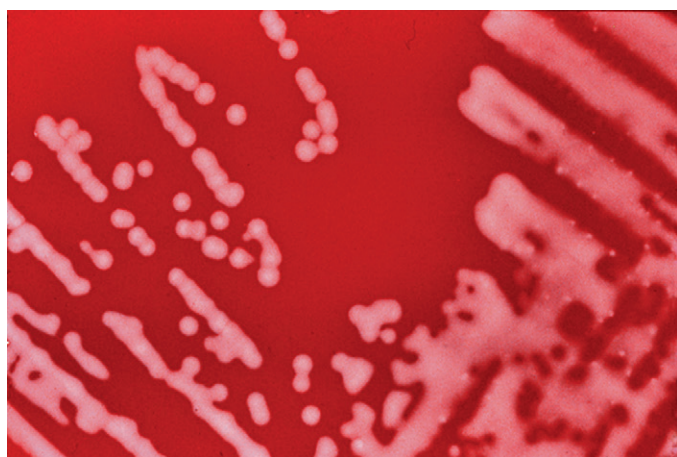


Fig. 21.13 *Achromobacter xylosoxidans* on sheep blood agar.

name). All species reduce nitrates to nitrites; *A. xylosoxidans* and *A. faecalis* can further reduce nitrites to nitrogen gas.

The asaccharolytic members of the group, *A. faecalis* and *A. piechaudii*, are usually susceptible to SXT, piperacillin, ticarcillin, ceftazidime, and quinolones (although variability occurs). Resistance to aztreonam and the aminoglycosides is common. Some strains of *A. piechaudii* may be susceptible to amoxicillin. *A. xylosoxidans* is usually resistant to aminoglycosides, ampicillin, first- and second-generation cephalosporins, chloramphenicol, and fluoroquinolones, but is usually susceptible to piperacillin, third-generation cephalosporins, carbapenems, and SXT. Resistance to all of these antimicrobial agents has been increasing, however, especially in immunocompromised patients in whom *A. xylosoxidans* is isolated.

Brevundimonas

Brevundimonas spp. are infrequent isolates in clinical microbiology laboratories. *Brevundimonas diminuta* has been found in blood, CSF, urine, and wounds; it is often considered a contaminant. A clinical isolate in an immunocompromised patient, however, was recently considered to be a pathogen. The isolate carried a VIM-2-metallo- β -lactamase, contributing to its multidrug resistance. *B. diminuta* isolates are motile and possess a single polar flagellum, oxidize glucose, and are oxidase positive. Most strains grow on MAC agar. In vitro, the organism demonstrates resistance to ampicillin, cefoxitin, and nalidixic acid; intrinsic resistance to fluoroquinolones has been detected.

B. vesicularis has been reported as a cause of meningitis, infective endocarditis, and infections in a CAPD patient; it was also isolated, with unknown significance, from urine and eye specimens. In 2012, there was a report of eight neonates in one neonatal ICU who had *B. vesicularis* septicemia, and three of the eight neonates also had meningitis caused by the organism. In addition, other cases of bacteremia have been described. Laboratory scientists should realize that *Brevundimonas* can be a significant pathogen. Like *B. diminuta*, *B. vesicularis* is a slender rod, with polar flagella. Only about 25% of *B. vesicularis* strains will grow on MAC agar. Most strains of *B. vesicularis* produce an orange intracellular pigment. *B. vesicularis* is also oxidase positive and oxidizes glucose and maltose. Esculin hydrolysis is the best test to differentiate the two species; approximately 88% of *B. vesicularis* isolates are positive, whereas *B. diminuta* isolates are rarely positive. *B. vesicularis* has been described as susceptible to fluoroquinolones and

piperacillin-tazobactam, but resistant to carbapenems, aztreonam, and the cephalosporins.

CDC groups EO-3, EO-4, and *Paracoccus*

The taxonomy of Centers for Disease Control and Prevention (CDC) groups EO-2, EO-3, and EO-4 is unclear; EO refers to **eugonic oxidizer**. EO-2 has been named *Paracoccus yeei*. Isolates of *P. yeei* have been recovered from blood cultures and wound infections and a cutaneous bulla in a 67-year-old male patient. It was also found in at least one case of uveitis, for which it was considered the potential pathogen. It has been seen in infections following keratoplasty, myocarditis in a heart transplant patient, and infection associated with CAPD. EO-3 has been reported in a case of CAPD infection. Isolates of EO-3 and EO-4 have been obtained from urine, eye discharge, blood, pleural fluid, and CSF and from lung, throat, and genitourinary tract specimens, in which their clinical significance remains unclear.

Isolates of *P. yeei*, EO-3, and EO-4 are oxidase-positive, nonmotile, saccharolytic coccobacilli that grow weakly, if at all, on MAC agar. They all oxidize glucose and xylose, but differ in oxidation of lactose and mannitol. EO-3 and many EO-4 isolates have a yellow nondiffusible pigment. *P. yeei* is further characterized by the production of characteristic coccoid or O-shaped cells on Gram stain, the latter of which results from the presence of vacuolated or peripherally stained cells. Susceptibility to antimicrobial agents is not well known.

Chromobacterium

Chromobacterium violaceum is the only clinically relevant species in the genus. The reservoirs are soil and water; isolates are more commonly found in tropical and subtropical climates, particularly in Southeast Asia and India. *C. violaceum* is an opportunist, attacking immunocompromised patients with neutrophil deficits, including CGD, usually as a result of contamination of wounds with water or soil. It has been isolated from cases of osteomyelitis, abscesses, and septicemia as well as from urine and gastrointestinal infections. A skin lesion is the typical portal of entry.

C. violaceum is a fermentative, gram-negative bacillus that might be oxidase positive, so it may be mistaken initially as a nonfermenter in the laboratory. It is motile with polar flagella and, as its name implies, produces a violet pigment about 91% of the time. The pigment is violacein, an ethanol-soluble, water-insoluble pigment. The presence of the pigment can hamper proper oxidase reactions. Isolates are usually motile, catalase positive, and indole negative (nonpigmented strains may be indole positive). Isolates ferment glucose and, variably, sucrose; they grow on MAC agar and most enteric media, reduce nitrate, and grow at 42°C. On Gram stain, the organisms may appear as curved bacilli that resemble vibrios, which are also oxidase positive. Nonpigmented strains have been confused with *Aeromonas* spp.

In 2011, 106 patients with *C. violaceum* infections were reported in a clinical review article. Fever, sepsis, skin lesions, and abdominal pain were the most common presenting symptoms, and the liver was the most common site of localized abscesses. The most important risk factors for death in *C. violaceum* bacteremia were young age, presence of localized abscess, shorter clinical course, and inappropriate

antimicrobial treatment. Relapse and reinfections also seemed to occur with these infections. Most isolates of *C. violaceum* are sensitive to fluoroquinolones, tetracyclines, carbapenems, gentamicin, and SXT but resistant to β -lactam antibiotics.

Comamonas and *Delftia*

Comamonas spp. and *Delftia* spp. are straight to slightly curved rods, produce alkalinity in OF media, are catalase and oxidase positive, are usually motile by multitrichous polar flagella, and reduce nitrate to nitrite. Ubiquitous in soil and water, they are rarely isolated from clinical specimens but have been found in hospital equipment and fluids. It is phenotypically difficult to distinguish among the *Comamonas* spp.; therefore isolates are often reported as *Comamonas* spp.

Isolates of *C. testosteroni* and *C. terrigena* have been reported to cause nosocomial bacteremia. In 2011 a review of the literature found 28 cases of *C. testosteroni* infection and a total of seven bacteremias. One patient receiving hemodialysis experienced *C. testosteroni* bacteremia accompanied by endocarditis and signs of peripheral embolism. The case had a fatal outcome.

Delftia acidovorans has been associated with keratitis in soft contact lens wearers and nosocomial infections including bacteremia and endocarditis. *D. acidovorans* can oxidize fructose and mannitol like the *Comamonas* spp. Some strains produce a fluorescent pigment. Isolates of *D. acidovorans* are usually aminoglycoside resistant. In cases of *Pseudomonas*-like infections of the eye, aminoglycosides are often the first-line therapy; therefore it is important to obtain the correct identification as quickly as possible and/or its antimicrobial susceptibility for appropriate treatment. *Delftia tsuruhatensis* has been associated with catheter-related bacteremia. This isolate was identified incorrectly as *D. acidovorans* with the VITEK 2 card; however, it was identified correctly with 16S rRNA sequencing. Disk diffusion susceptibility testing revealed the isolate to be susceptible to fluoroquinolones, carbapenems, third-generation cephalosporins, and piperacillin-tazobactam, but resistant to ampicillin, aminoglycosides, and colistin.

Weeksellaceae

About 15 genera, including *Bergeyella*, *Chryseobacterium*, *Elizabethkingia*, *Empedobacter*, *Weeksella*, and *Wautersiella*, are placed in the family Weeksellaceae. Members of the family are ubiquitous in soil and water and are not considered part of the normal human microbiota. Because isolates often contaminate hospital equipment, they can be important causes of nosocomial infections. Even though the organisms are weak fermenters, the reactions are usually delayed, and the isolates initially appear to be nonfermenters.

Most diseases produced by members of the Weeksellaceae are caused by *Elizabethkingia meningoseptica*. The infection typically presents as meningitis or septicemia in a newborn, especially in conjunction with prematurity. In adults, *E. meningoseptica* can cause pneumonia, endocarditis, bacteremia, and meningitis, especially in critically ill patients. There have been a few documented outbreaks of *E. meningoseptica* infections subsequent to the use of materials that had become contaminated with the organism. Tissue allograft material was found to be the cause of infection in two recipients during repair of their cruciate ligament; the allografts were

thought to have become contaminated during processing of the tissue. In 2011, the U.S. Food and Drug Administration (FDA) initiated a recall of povidone-iodine pads used for skin disinfection because they were found to be contaminated with *E. meningoseptica*. The bacteria produce acid from glucose, maltose, and mannitol. They hydrolyze esculin and are indole positive, and growth on MacConkey agar is variable.

The genus *Chryseobacterium* contains over 100 species. *Chryseobacterium indologenes* is the most frequently isolated species in this genus found in human specimens, although it is often considered insignificant when isolated in rare numbers or from only one medium or one specimen. It has, however, been linked to healthcare-associated infections, including bacteremia, usually in immunosuppressed patients or patients who have been receiving long-term antimicrobial therapy, and in ocular infections. Isolated cases of endophthalmitis have been reported to be caused by *Empedobacter brevis* following cataract surgery. The source was thought to be poor sterilization procedures.

Members of the genus *Wautersiella* have been isolated from blood and surgical wounds. Isolates closely resemble *E. brevis*. *Weeksella virosa* has been found in genitourinary specimens and will grow on modified Thayer-Martin medium or other media selective for *Neisseria gonorrhoeae*. *Bergeyella zoohelicum* has been isolated from cases of cellulitis, tenosynovitis, septicemia, pneumonia, and meningitis, particularly in association with dog and cat bite wounds, because it is part of their normal oral biota. However, there have been cases of bacteremia without any known animal exposures. *Weeksella* isolates are asaccharolytic, indole and oxidase positive, and fail to grow on MAC agar.

Most organisms in the family Weeksellaceae are nonmotile, and many species possess an intracellular pigment that can be salmon-pink or pale yellow (Fig. 21.14). On media with blood, a lavender-green discoloration of the agar may occur because of the proteolytic activity of the organisms. Some species release a characteristic fruity odor. Most isolates are DNase, oxidase, gelatin hydrolysis, and weakly indole positive (the more sensitive Ehrlich indole test is recommended over the Kovacs test). Microscopically, *E. meningosepticum* and *C. indologenes* are long, thin bacilli, often with bulbous ends.

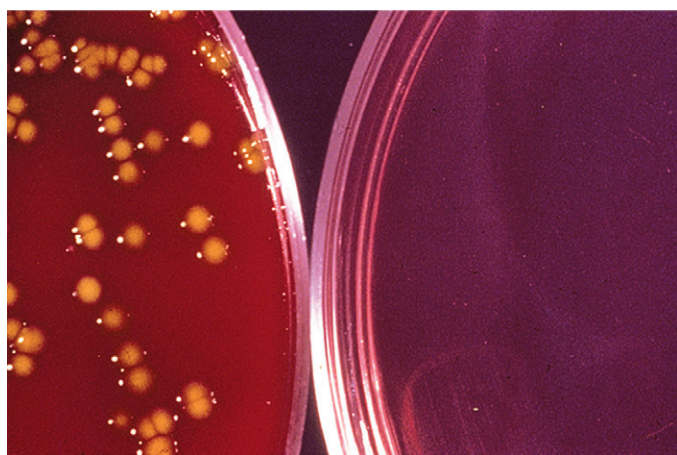


Fig. 21.14 *Elizabethkingia meningoseptica*. Note the growth with yellow pigment on sheep blood agar (left) and absence of growth on MacConkey agar plate (right).

In vitro, most species are resistant to aminoglycosides and β -lactam antibiotics, but some species are susceptible to vancomycin, which is an unusual characteristic for gram-negative bacilli. However, one report of susceptibility testing of more than 50 isolates of *Chryseobacterium* spp. demonstrated reduced activity to vancomycin compared with what had been previously reported. Activity to SXT, fluoroquinolones, and piperacillin-tazobactam is often good. *Balneatrix* is resistant to clindamycin and vancomycin, unlike *E. meningoseptica*. *Weeksella* and *Bergeyella* spp. are susceptible to penicillin and other antimicrobial agents.

Methylobacterium* and *Roseomonas

The genus *Methylobacterium*, in the family Methylobacteriaceae, contains over 50 named species plus additional unnamed biovars. Isolates produce a characteristic pink to coral pigment and can use methanol as a sole source of carbon and energy. Epidemiologically, the *Methylobacterium* are isolated from soil, vegetation, sewage, water, and hospital nebulizers. They have also been recovered from clinical specimens such as throat swabs, bronchial washes, and even blood specimens. Clinically, these organisms have been reported to cause bacteremia, peritonitis, synovitis, and skin ulcers, usually in immunocompromised hosts. Contaminated tap water has been implicated as a cause of positive blood cultures in a patient receiving irrigations who had recently undergone bone marrow transplant. A leukemic patient who had undergone stem cell transplant was found to have bacteremia with *Methylobacterium fujisawaense*.

Methylobacterium mesophilicum and *M. zatmanii* are the two species usually isolated in clinical specimens. They prefer a lower temperature (25° to 35°C), produce distinctive large vacuolated pleomorphic rods, and are oxidase variable and motile with a polar flagellum. Oxidation of carbohydrates is weak. Isolates are slow growers, producing 1-mm dry, coral, or pink colonies in 4 to 5 days. They are often first seen on fungal media, such as Sabouraud dextrose agar, and do not grow as well on SBA, chocolate, modified Thayer-Martin, or buffered charcoal-yeast extract agars. No growth on MAC agar is typical.

The other pink-pigmented, nonfermentative bacilli are a group of bacteria now classified in the genus *Roseomonas*, in the family Acetobacteraceae. These bacteria have been isolated from the environment and clinical specimens, including blood, CSF, sputum, abscess, and wound specimens. In a review covering 10 years, 17 of 20 patients from whom *Roseomonas* spp. were isolated were considered to have true infection, primarily in immunocompromised patients. *R. mucosa* was the usual isolate, and catheter-related bloodstream infection was the most common infection seen. On Sabouraud dextrose agar, *Roseomonas* spp. produce pink, mucoid, almost runny colonies (Fig. 21.15); however, they do not appear black under long-wavelength ultraviolet light, as do *Methylobacterium* spp. *Roseomonas* spp. are nonvacuolated, coccoid bacteria, forming pairs and short chains. They are variable in the oxidase reaction, often weakly positive, and urease positive. Unlike *Methylobacterium* spp., species of *Roseomonas* will usually grow on MAC agar. Although uncommon isolates, they are the most common pink-pigmented, gram-negative, nonfermentative bacillus recovered in clinical laboratories.

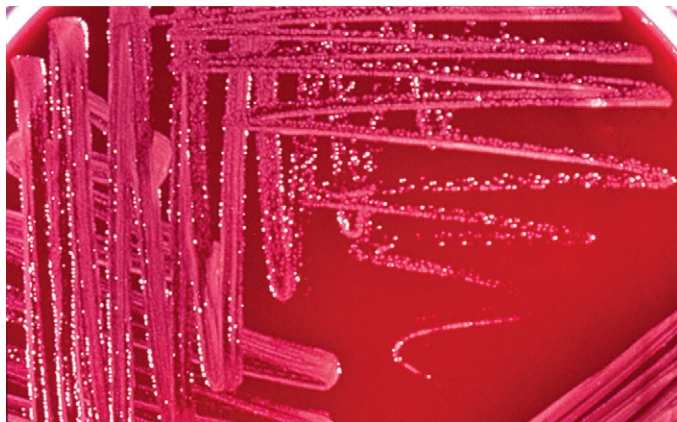


Fig. 21.15 *Roseomonas* on sheep blood agar. Note the pink to red colonies.

Ralstonia and *Cupriavidus*

Ralstonia and *Cupriavidus* belong to the family Burkholderiaceae along with *Burkholderia* and numerous other genera. *Ralstonia pickettii* is the most common species of the seven in this genus isolated from humans. Isolates can be found contaminating sterile hospital fluids and, as such, may be isolated from specimens including urine, nasopharynx, abscess, wound, and blood specimens, usually as colonizers or contaminants. It has been linked to meningitis, endocarditis, and osteomyelitis. *R. pickettii* and *Ralstonia mannitolilytica* have been isolated from respiratory specimens of patients with CF and in non-CF patients as well. The significance is not always clear, although it appears that colonization increases in patients who are mechanically ventilated compared with patients who are not. *R. mannitolilytica* appears to be the *Ralstonia* species most often causing infections in patients with CF. A case of community-acquired lobar pneumonia and pulmonary abscess was described in a 65-year-old patient; the lung biopsy specimen and thoracentesis fluid grew *R. pickettii*, and symptoms resolved after cephalosporin and carbapenem were administered for 3 weeks.

R. pickettii isolates are slow growers, requiring more than 72 hours on primary cultures before colonies are visible. *R. pickettii* is oxidase and catalase positive (there are reports of rare catalase-negative strains), grows on MAC agar, reduces nitrate, oxidizes glucose and xylose, and is motile by means of a single polar flagellum. There are many species of *Ralstonia*, and definitive identification may require methods other than phenotypic methods, although there are even some problems with those.

Cupriavidus pauculus has been isolated from a number of aquatic environments and has been recognized as an opportunistic pathogen that can cause serious infections, including septicemia, peritonitis, abscesses, and tenosynovitis, most notably in immunocompromised patients. There was a pseudobacteremia cluster involving five patients, four adults in the emergency department and one neonate in the ICU. None appeared to have symptoms of a bloodstream infection. All isolates had the same molecular fingerprinting; environmental sources were presumed to have caused the contamination, although none were found. The unusual practice of moistening culturette swabs with tap water before the collection of microbiology samples led to an outbreak of *C. pauculus*

infection in an outpatient clinic in Columbus, Ohio. *C. pauculus* and *Cupriavidus gilardi* have also been isolated from patients with CF.

C. pauculus is a motile (peritrichous flagella), oxidase-positive, catalase-positive, asaccharolytic, gram-negative bacillus. Most strains grow on MAC agar. Isolates are resistant to aminoglycosides, ampicillin, and first- and second-generation cephalosporins. They are usually susceptible to quinolones, third-generation cephalosporins, piperacillin, and doxycycline, although not many isolates have been tested.

Shewanella

Shewanella is one of about four genera in the family Shewanellaceae. *Shewanella algae* and *S. putrefaciens*, though infrequent isolates and rarely pathogenic, have been recovered from various human specimens, including specimens from abscesses and traumatic ulcers, otitis media, ocular infections, osteomyelitis, peritonitis, and septicemia, but are usually present in mixed culture. Frequently, they probably represent colonization rather than infection. Environmental sources, such as stagnant water, natural gas (petroleum), brine, and spoiled dairy products, meat, and fish may contain *S. putrefaciens*. *S. algae* is more frequently isolated from clinical specimens than *S. putrefaciens*, whereas *S. putrefaciens* is more frequently isolated from environmental sources.

Colonies of *Shewanella* spp. are often mucoid and can produce a tan to brown pigment causing greenish discoloration of SBA. Both species are motile, ornithine decarboxylase and nitrate reductase positive, and produce profuse H_2S in TSIA, resembling H_2S producers of the order Enterobacterales. The positive oxidase test should differentiate *Shewanella* from the order Enterobacterales (except for *Plesiomonas*). *S. algae* requires NaCl (halophilic) and is asaccharolytic, whereas *S. putrefaciens* is nonhalophilic and saccharolytic. These organisms are usually susceptible to ampicillin, tetracycline, chloramphenicol, erythromycin, and the aminoglycosides, but resistant to penicillin and cefazolin.

Sphingomonas

Sphingomonas paucimobilis can be isolated from many water sources, including swimming pools, as well as from hospital equipment and laboratory supplies. The genus *Sphingomonas* contains over 100 species, but only two are believed to be clinically significant: *S. paucimobilis* and *S. parapaucimobilis*. Documented *S. paucimobilis* infections include peritonitis associated with CAPD, septicemia, meningitis, leg ulcers, empyema, and splenic and brain abscesses. *S. parapaucimobilis* has been isolated from sputum, urine, and vaginal specimens. Although isolates have been found to produce esterases, endotoxin, lipases, and phosphatases, inherent virulence is limited, and most isolates should be considered as colonizers or contaminants initially unless repeatedly isolated from a patient's samples. In a study of 55 documented infections with *S. paucimobilis* in Taiwan, 53% were community-acquired infections, and in this group almost 50% presented as bacteremia. In the hospital-associated infections, most presented with pneumonia.

The yellow-pigmented *S. paucimobilis* does not grow on MAC agar and requires more than 48 hours for culture on SBA (Fig. 21.16). Isolates are weakly oxidase positive (some strains

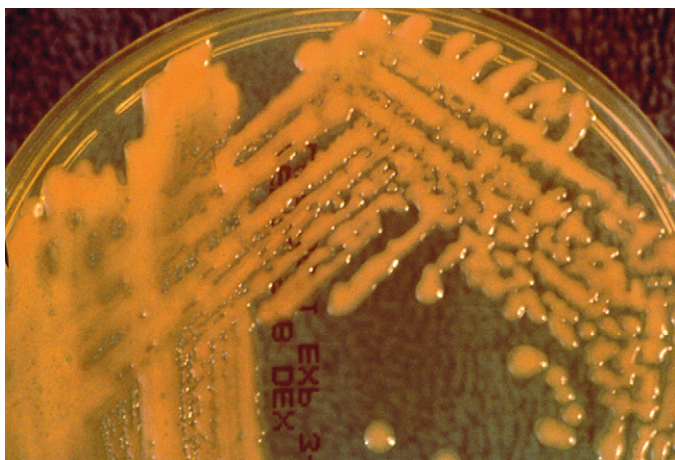


Fig. 21.16 *Sphingomonas paucimobilis* on sheep blood agar. Note the yellow colonies.

may be negative), motile at 18° to 22°C but not at 37°C, indole negative, and oxidizers. *S. parapaucimobilis* resembles *S. paucimobilis*, except that isolates of *S. parapaucimobilis* are H₂S positive by the lead acetate method, Simmon citrate positive, and DNase negative. *S. paucimobilis* isolates demonstrate variable resistance to antimicrobial agents, although most are susceptible to the aminoglycosides, tetracyclines, chloramphenicol, and SXT. Susceptibility to the third-generation cephalosporins (e.g., ceftazidime, ceftriaxone, ceftizoxime) and to the fluoroquinolones differs. Most isolates are resistant to colistin. They are susceptible to polymyxin B, which differentiates isolates from members of the genus *Sphingobacterium*, which they resemble. They are typically sensitive to vancomycin, which is unusual for gram-negative bacteria.

POINTS TO REMEMBER

- Nonfermenters will not acidify the butt of TSIA or KIA.
- Most nonfermenters are oxidase positive, a key test in differentiation from most members of the order Enterobacterales.
- Nonfermenters are environmental isolates that rarely cause disease in healthy humans.
- Nonfermenters can be more resistant to antimicrobial agents than members of the order Enterobacterales. There are specific methods and breakpoints available for many of the nonfermenters in the CLSI standards.
- The most common nonfermenters isolated in clinical microbiology laboratories are *P. aeruginosa*, *A. baumannii* complex, *S. maltophilia*, and *B. cepacia* complex.
- *Acinetobacter* spp. may retain the crystal violet stain resembling gram-positive bacteria.
- *Pseudomonas aeruginosa* produces several virulent factors leading to its pathogenicity, and many strains produce a fruity, grapelike odor.
- Phenotypic identification will not identify all nonfermenters; MALDI-TOF mass spectrometry or 16S rRNA DNA sequencing is required if confirmation is needed.
- The decision to identify many of the nonfermenters is based on the site from which they have been isolated, that is, whether they have been recovered from a normally sterile

site in pure culture or from a nonsterile site in which three or four other bacterial species are also present.

- Specimens from patients with CF often demonstrate a wide variety of nonfermentative bacilli and may require more definitive identification methods. The most common, however, is *P. aeruginosa*.
- Melioidosis is most common in areas of the world with tropical and subtropical climates. Most cases in the United States occur in persons returning from a country where the disease is endemic, although recent cases with no travel history have been reported.

LEARNING ASSESSMENT QUESTIONS

1. Which of the following characteristic clues can indicate the presence of a nonfermenter in the clinical laboratory?
 - a. Oxidase-positive reaction that can be weak
 - b. No acid production in the slant or butt of TSIA or KIA
 - c. Resistance to a variety of classes of antimicrobial agents
 - d. All of the above
2. What is the difference between nonfermentative and fermentative organisms based on carbohydrate utilization?
 - a. Nonfermenters are able to ferment sugars.
 - b. Nonfermenters are able to acidify the butt of TSI or KIA.
 - c. Fermenters are not able to acidify the butt of TSI or KIA.
 - d. Fermenters are able to metabolize carbohydrates.
3. Which of the following is the typical natural habitat of most nonfermenters?
 - a. All nonfermenters are part of the normal human biota.
 - b. Nonfermenters are never found in soil and water.
 - c. Most nonfermenters often exist in a moist or aquatic environment.
 - d. Nonfermenters are only found in hospital environments.
4. Which type of hospital-acquired infections do nonfermenters cause?
 - a. Urinary tract infections
 - b. Postsurgical wound infections
 - c. Septicemia
 - d. All of the above
5. Which of the following is a risk factor for diseases caused by nonfermentative gram-negative bacilli?
 - a. Immunosuppression
 - b. Foreign-body implantation
 - c. Trauma
 - d. All of the above
6. Which of the following is the most common and clinically significant nonfermenter associated with clinical infections?
 - a. *Acinetobacter baumannii*
 - b. *Stenotrophomonas maltophilia*
 - c. *Pseudomonas aeruginosa*
 - d. *Burkholderia* spp.
7. What are the four most common nonfermentative, gram-negative bacilli isolated in the clinical laboratory?
8. What are the typical susceptibility patterns of the most commonly isolated nonfermenters?
9. Which initial clues indicate that an isolate is a nonfermenter?
 - a. Nonfermenters will not grow on media selective for gram-negative bacilli.

- b. Nonfermenters are usually lactose-positive on media selective for gram-negative bacilli.
 - c. Nonfermenters will be oxidase-negative.
 - d. Nonfermenters will not produce an acid (yellow) butt in TSI or KIA.
10. How can *Pseudomonas aeruginosa* be differentiated from other fluorescent group pseudomonads?
 - a. Ability to grow at 42°C
 - b. Presence of a blue-green pigment diffusing through the medium
 - c. Grapelike odor
 - d. All of the above
 11. Which of the following is an identifying characteristic of *Acinetobacter* spp.?
 - a. Gram-positive coccobacillary organisms
 - b. Oxidase-positive
 - c. Have a bluish-purple appearance on MacConkey agar
 - d. Catalase negative
 12. Which nonfermenter is most commonly isolated from patients with cystic fibrosis?
 - a. *Burkholderia cenocepacia*
 - b. *Pseudomonas aeruginosa*
 - c. *Acinetobacter baumannii*
 - d. *Burkholderia multivorans*
 13. Blood cultures from a 45-year-old female patient who has been receiving treatment for breast cancer grow an oxidase-negative, motile, gram-negative bacillus. The organism grows well on a blood agar plate and produces clear, colorless colonies on MacConkey agar and on TSI, K/K reaction. Based on these characteristics, which of the following organisms should be suspected?
 - a. *Acinetobacter baumannii*
 - b. *Stenotrophomonas maltophilia*
 - c. *Pseudomonas aeruginosa*
 - d. *Burkholderia* spp.
 14. Which of the following organisms causes melioidosis, a glanders-like disease in humans and animals?
 - a. *Burkholderia pseudomallei*
 - b. *Pseudomonas aeruginosa*
 - c. *Acinetobacter baumannii*
 - d. *Burkholderia multivorans*
 15. An organism is isolated from the endotracheal tube of a patient receiving chemotherapy. The gram-negative coccobacillus grows on a blood agar plate and produces clear, colorless colonies on MacConkey agar. The isolate is nonmotile, oxidase negative, and biochemically inactive. Which of the following organisms should be suspected?
 - a. *Burkholderia pseudomallei*
 - b. *Pseudomonas aeruginosa*
 - c. *Acinetobacter lwoffii*
 - d. *Stenotrophomonas maltophilia*

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Anaerobes of clinical importance

Nancy Gouin

CHAPTER OUTLINE

Important concepts in anaerobic bacteriology, 497

- Anaerobes defined, 497
- Why some organisms are anaerobes, 498
- Where anaerobes are found, 498
- Anaerobes at specific anatomic sites, 498
- Factors that predispose patients to anaerobic infections, 500
- Indications of anaerobe involvement in human disease, 500

Frequently encountered anaerobes and their associated diseases, 501

- Gram-positive, spore-forming anaerobic bacilli, 501
- Gram-positive, non-spore-forming anaerobic bacilli, 503
- Anaerobic gram-negative bacilli, 504
- Anaerobic cocci, 504

Specimen selection, collection, transport, and processing, 505

- Specimen quality, 505
- Specimen transport and processing, 505
- Processing clinical samples for recovery of anaerobic pathogens, 506

Procedures for identifying anaerobic isolates, 512

- Preliminary procedures, 513
- Indications of the presence of anaerobes in cultures, 513
- Presumptive identification of clinically significant anaerobes, 514
- Definitive identification of anaerobic isolates, 518
- Identification of *Clostridium* and *Clostridioides* species, 521
- Identification of anaerobic non-spore-forming, gram-positive bacilli, 523
- Identification of anaerobic gram-negative bacilli, 524
- Identification of anaerobic cocci, 526

Antimicrobial susceptibility testing, 527

- Problems in susceptibility testing of anaerobic isolates, 528

Treatment of anaerobe-associated diseases, 529

- Surgical therapy, 529
- Hyperbaric oxygen, 529
- Antimicrobial therapy, 529
- Antitoxins, 529

Fecal microbiota transplant, 529

Bibliography, 531

OBJECTIVES

After reading and studying this chapter, you should be able to:

1. Characterize anaerobic bacteria, including why they are sensitive to oxygen.
2. Describe where anaerobes might be found in the environment and human body.
3. Differentiate the various types of anaerobes with regard to atmospheric requirements (i.e., obligate anaerobes, facultative anaerobes, and aerotolerant anaerobes).
4. Discuss how anaerobes, as part of endogenous microbiota, initiate and establish infection.
5. Describe the endogenous anaerobes commonly involved in human infections and the types of infections with which they are most often associated.
6. Assess specimens that are acceptable and unacceptable for anaerobic culture.
7. Describe the atmospheric requirements, isolation media, and identification systems used for the culture and isolation of anaerobes.
8. Given the signs and manifestations of an anaerobic infection, identify the most probable causative agent of:
 - Wound botulism
 - Tetanus
 - Gas gangrene
 - Actinomycosis
 - Pseudomembranous colitis
 - Bacterial vaginosis
9. Compare the methods available for cultivation of obligate anaerobes.
10. Compare the microscopic and colony morphology of anaerobic isolates.
11. Evaluate the different systems used to grow obligate anaerobes in the clinical microbiology laboratory.
12. Given the results of key biochemical laboratory tests, identify the most likely anaerobic isolate.

13. Evaluate the laboratory methods for the diagnosis of *Clostridioides difficile* infection.
14. Name the pigment-producing anaerobic bacteria.
15. Discuss the acceptable susceptibility methods for testing of anaerobes for antimicrobial susceptibility and when anaerobe susceptibility testing should be performed.
16. Describe the antimicrobial agents to be tested for susceptibility and resistance patterns of anaerobic organisms.
17. Describe the five major approaches to treat anaerobe-associated diseases: antimicrobial therapy, surgical therapy, hyperbaric oxygen therapy, administration of antitoxins, and fecal microbiota transplant.

KEY TERMS

Actinomycosis	Enteritis necroticans
Aerotolerance test	Exogenous anaerobe
Aerotolerant anaerobe	Facultative anaerobe
Anaerobe	Hydroxyl radical
Anaerobic chamber	Microaerophilic
Bacterial vaginosis (BV)	Myonecrosis
Botulism	Obligate anaerobe
Capnophilic	Pseudomembranous colitis
Catalase	Superoxide anion
<i>Clostridioides difficile</i>-associated disease (CDAD)	Superoxide dismutase
Endogenous microbiota	Tetanus

Case in point

A 45-year-old male patient who worked as a farmer was admitted to the hospital for complications resulting from a tractor accident that caused traumatic injury to his left leg. The patient's leg was painful, bluish, and edematous. An x-ray revealed pockets of gas in the tissue. A complete blood count revealed a marked increase in the levels of neutrophils and a total white blood cell count of 33,000/mL. A Gram-stained smear of the wound specimen revealed numerous large, rectangular-shaped, gram-positive bacilli, with no spores and very few leukocytes. The patient was immediately scheduled for surgical debridement and was given a broad-spectrum antimicrobial agent. Within 24 hours, anaerobic cultures grew gram-positive rods, with a double zone of β hemolysis surrounding the colonies on sheep blood agar (SBA) incubated anaerobically.

Issues to consider

After reading the patient's case history, consider:

- The clinical significance of anaerobes in human infections
- Indications of an anaerobic infection
- The importance of proper selection, collection, transport, and processing of anaerobic specimens
- Various anaerobes that may be found in different specimen types
- Extent of anaerobe identification
- When and how to perform susceptibility testing of anaerobes

Anaerobic bacteria are important in human and veterinary medicine because they play a role in serious and potentially fatal infections and intoxications. They are involved in infectious

processes in almost every organ or tissue of the body and consequently can be recovered from most clinical specimens. This chapter discusses how anaerobes differ from aerobic bacteria, the importance of anaerobes as endogenous microbiota and their role as disease-causing agents, the proper techniques for recovery and identification of anaerobes, susceptibility testing of anaerobic isolates, and treatment of anaerobic infections.

Important concepts in anaerobic bacteriology

Anaerobes defined

An **anaerobe** is a bacterium able to replicate in the absence of oxygen. To recover all potential pathogens, the clinical microbiology laboratory must use a variety of atmospheric conditions for culturing bacteria (Table 22.1). Ambient air contains approximately 21% oxygen and 0.04% carbon dioxide (CO₂). Obligate, or strict, aerobes require oxygen for metabolism, and they can grow well in an ambient air incubator. **Capnophilic** organisms, such as *Capnocytophaga*, grow best when the concentration of CO₂ is increased to the range of 5% to 10% in a CO₂ incubator. This increase in CO₂ concentration reduces the oxygen concentration to 15%, which is still sufficient to allow aerobic organisms to replicate. **Microaerophilic** organisms, such as *Campylobacter*, require the oxygen concentration to be reduced to 5% or less. **Facultative anaerobes**, such as *Escherichia coli* and *Staphylococcus aureus*, preferentially use oxygen as an electron acceptor if it is available but can grow in the absence of oxygen, albeit at a slower rate.

Table 22.1 Classification of microorganisms on the basis of their relationship to oxygen and carbon dioxide

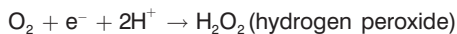
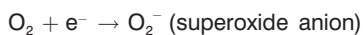
Category	Requirement	Examples
Obligate aerobe	15%–21% O ₂ (as found in a CO ₂ incubator or ambient air)	Mycobacteria, fungi
Microaerophile	5% O ₂	<i>Campylobacter</i> , <i>Helicobacter</i>
Facultative anaerobe	Multiplies well in the presence or absence of O ₂	Enterobacterales, most staphylococci
Aerotolerant anaerobe	Reduced concentrations of O ₂ (anaerobic system and microaerophilic environments)	Most strains of streptococci, <i>Propionibacterium</i> , <i>Lactobacillus</i> , some <i>Clostridium</i>
Obligate anaerobe	Strict anaerobic environment (0% O ₂)	Most <i>Bacteroides</i> spp., many species of <i>Clostridium</i> , <i>Eubacterium</i> , <i>Fusobacterium</i> , <i>Peptostreptococcus</i> , <i>Porphyromonas</i>
Capnophile	5%–10% CO ₂	Some anaerobes, <i>Neisseria</i> , <i>Capnocytophaga</i>

Modified from Engelkirk, P. G., et al. (1992). *Principles and practice of clinical anaerobic bacteriology*. Belmont, CA: Star Publishing.

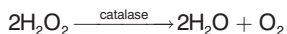
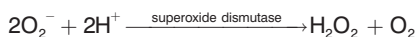
Anaerobes differ in their response to oxygen. Some anaerobes are killed almost immediately in the presence of oxygen (e.g., *Clostridium novyi*). These are referred to as **obligate**, or **strict, anaerobes**. To grow obligate anaerobes, the laboratory must use oxygen-free growth conditions. This can be accomplished by a variety of mechanisms (see the “Processing Clinical Samples for Recovery of Anaerobic Pathogens” section later in this chapter). Other anaerobes can survive a short exposure to oxygen but typically will not be able to perform metabolic processes unless placed into an anaerobic environment. These organisms are referred to as **aerotolerant**, or **moderate, anaerobes**. Many anaerobic pathogens encountered in the clinical microbiology laboratory fall into this group (e.g., *Bacteroides fragilis*).

Why some organisms are anaerobes

Molecular oxygen can be toxic to some anaerobes, but substances produced when oxygen becomes reduced through metabolic processes are even more toxic. During oxidation-reduction reactions that occur during normal cellular metabolism, molecular oxygen is reduced to **superoxide anion** (O_2^-) and hydrogen peroxide (H_2O_2) in a stepwise manner by the addition of electrons, as shown in the following equations:



Furthermore, one hypothesis of oxygen toxicity proposes that the superoxide anion reacts with hydrogen peroxide in the presence of iron (Fe^{3+}/Fe^{2+}) to generate the **hydroxyl radical** ($\cdot OH$). This short-lived molecule is the most potent biological oxidant known. The reaction between the hydroxyl radical and superoxide anion forms singlet oxygen, which is also damaging to cells. Together, these toxic compounds are detrimental to cell components such as proteins and nucleic acids. It is obvious that cells need a way to remove these harmful molecules if they are to survive in the presence of oxygen. Strict aerobic and facultative anaerobic bacteria that use oxygen have the enzymes **superoxide dismutase** and/or **catalase** to protect them from superoxide anions and their toxic derivatives, as shown in the following equations:



Superoxide dismutase converts the superoxide anion to oxygen and hydrogen peroxide. Hydrogen peroxide can be toxic to cells but not to the degree of the superoxide anion or hydroxyl radical. Hydrogen peroxide will diffuse out from the cell, but many organisms also possess the enzyme catalase, which breaks hydrogen peroxide down to oxygen and water, thereby negating its toxic effect. Because the hydroxyl radical is a product of the further reduction of the superoxide anion, elimination of the superoxide anion by superoxide dismutase will inhibit the formation of the hydroxyl radical.

Anaerobes, on the other hand, are particularly susceptible to these toxic derivatives of oxygen because they lack the protective enzymes superoxide dismutase and/or catalase, or the enzymes are present in low concentrations. Extended

exposure to oxygen results in cell death for strict anaerobes and cessation of growth for oxygen-tolerant anaerobes. Strict anaerobes also might require an environment that has a low oxidation-reduction (redox) potential. This may be in part because certain enzymes that are essential for bacterial growth require fully reduced sulfhydryl ($-SH$) groups to be active. Reducing agents such as thioglycollate, cysteine, and dithiothreitol often are added to microbiological media to obtain a low redox potential. In vivo, bacteria have a tendency to lower the redox potential at their site of growth. Consequently, anatomic sites colonized with mixtures of organisms, such as those found on mucosal surfaces, frequently provide favorable conditions for the growth of obligate anaerobes.

Where anaerobes are found

In a world in which oxygen abounds, anaerobes are found only in specific ecologic niches. They can be found in soil, in freshwater and saltwater sediments, and as components of the **endogenous microbiota** of humans and other animals. Anaerobes that exist outside the bodies of animals are referred to as **exogenous anaerobes**; the infections they cause are termed *exogenous infections*. Conversely, anaerobes that exist inside the body (endogenous microbiota) are termed *endogenous anaerobes* and are the source of endogenous infections.

Exogenous anaerobic infections are usually caused by gram-positive, spore-forming bacilli belonging to the genus *Clostridium*. Clostridia initiate infection when spores are ingested by way of contaminated food or gain access to the body through open wounds contaminated with soil. However, the anaerobes most frequently isolated from infectious processes in humans are those of endogenous origin. Table 22.2 shows endogenous anaerobes commonly encountered in human infections. Endogenous anaerobes can contribute to an infectious disease in any anatomic site of the body if suitable conditions exist for colonization and penetration of the bacteria.

Although many different species of anaerobes can be isolated from human clinical specimens, the number of species routinely isolated is relatively small. Table 22.3 lists the anaerobes most frequently recovered from clinical specimens at a university medical center, including members of the *B. fragilis* group, *Porphyromonas* and *Prevotella* spp., anaerobic cocci (e.g., peptostreptococci), and *Propionibacterium* spp. A pathogenic anaerobe frequently encountered in healthcare-associated infection is *Clostridioides difficile*, a cause of antibiotic-associated diarrhea.

Case check 22.1

In the Case in Point, the infecting organism was likely *Clostridium perfringens*, which can be found as exogenous microbiota in soil. The wound caused by the tractor accident was likely contaminated by soil containing the organism or its spores. The trauma to the legs following the accident allowed the organism to penetrate the skin surface.

Anaerobes at specific anatomic sites

Anaerobes outnumber aerobes on mucosal surfaces, such as the linings of the oral cavity, gastrointestinal (GI) tract, and genitourinary (GU) tract. These heavily colonized surfaces

are the usual portals of entry into the tissues and bloodstream for endogenous anaerobes. Under ordinary circumstances, microorganisms that are members of the microbiota do not cause disease, and many actually can be beneficial. However,

when some of these organisms gain access to usually sterile body sites, such as the bloodstream, brain, and lungs, they can cause serious or even fatal infections.

Knowledge of the composition of the microbiota at specific anatomic sites is useful for predicting the microorganisms most likely to be involved in infectious processes that arise at or adjacent to those sites. Table 22.4 summarizes the variety of endogenous anaerobes that may be found at specific body sites. Finding site-specific organisms at a distant and/or unusual site can serve as a clue to the underlying origin of an infectious process. For example, the isolation of oral anaerobes from a brain abscess may suggest invasion from the oral cavity, perhaps caused by poor dentition.

Skin

Indigenous members of the skin microbiota include anaerobes that colonize the sebaceous glands and hair follicles. *Cutibacterium acnes* is frequently isolated from blood cultures, but its presence often represents contamination from the patient's skin resulting from poor site preparation during the venipuncture. Nevertheless, *C. acnes* is considered an opportunistic pathogen and has been associated with cases of endocarditis, surgical wounds, and prosthetic joint infections. Other anaerobes found on the skin include gram-positive cocci (e.g., *Peptostreptococcus*). Superficial wound or abscess specimens aspirated by needle and syringe are much better specimens for anaerobic bacteriology than material collected by swabs; the latter often are contaminated with skin microbiota.

Respiratory tract

Of the bacteria present in saliva, nasal washings, and gingival and tooth scrapings, 90% are anaerobes. Gram-negative anaerobic bacilli (*Prevotella*, *Porphyromonas*, and *Fusobacterium*) and anaerobic cocci are the anaerobes occurring in the largest numbers. These anaerobes should be suspected as participants in any infectious process occurring in the oral cavity and in suspected cases of aspiration pneumonia. In addition,

Table 22.2 Endogenous anaerobes commonly involved in human infections

Infection	Anaerobe
Actinomycosis	<i>Actinomyces israelii</i> , other <i>Actinomyces</i> spp.
Antibiotic-associated diarrhea; pseudo-membranous colitis	<i>Clostridioides difficile</i>
Bacteremia	<i>Bacteroides fragilis</i> group, fusobacteria, clostridia, AGPC
Brain abscess	<i>Bacteroides</i> spp., <i>Prevotella</i> spp., <i>Porphyromonas</i> spp., <i>Fusobacterium</i> spp., <i>Clostridium</i> spp. (these infections are often polymicrobial)
Infections of the female genitourinary tract	AGPC, <i>Bacteroides</i> spp., <i>Clostridium</i> spp., <i>Prevotella bivia</i> , <i>Prevotella disiens</i> , <i>Porphyromonas</i> spp., <i>Actinomyces israelii</i> (IUD associated)
Intraabdominal infections, liver abscess, peritonitis, perineal and perirectal infections	<i>B. fragilis</i> group, other <i>Bacteroides</i> spp., <i>Fusobacterium</i> spp., <i>Clostridium perfringens</i> , other <i>Clostridium</i> spp., AGPC (frequently polymicrobial)
Myonecrosis	<i>C. perfringens</i> , <i>Clostridium novyi</i> , <i>Clostridium septicum</i> (80%–95% of the cases)
Head and neck infections, e.g., sinus, otitis media, dental abscesses, gingivitis, periodontitis	AGPC, <i>Porphyromonas</i> spp., <i>Fusobacterium</i> spp. (often polymicrobial)
Aspiration pneumonia, pleuropulmonary infections	<i>Porphyromonas</i> spp., <i>Fusobacterium nucleatum</i> , <i>Prevotella</i> spp., AGPC, <i>B. fragilis</i> group, <i>Actinomyces</i> spp.

AGPC, Anaerobic gram-positive cocci; IUD, intrauterine device.
Modified from Engelkirk, P. G., et al. (1992). *Principles and practice of clinical anaerobic bacteriology*. Belmont, CA: Star Publishing.

Table 22.3 Incidence of anaerobes at Scott and White Memorial Hospital^a

Organism group	Total	Blood isolates
<i>Bacteroides fragilis</i> group	377	16
<i>Fusobacterium</i>	47	4
<i>Prevotella</i> - <i>Porphyromonas</i> group	244	6
<i>Clostridium</i>	40	13
<i>Propionibacterium</i>	148	60 ^b
<i>Peptostreptococci</i>	155	6
<i>Veillonella</i>	7	0

^aData provided by authors.

^bMostly considered contaminants.

Table 22.4 Endogenous anaerobes of various anatomic sites

Site	Anaerobes
Skin	<i>Propionibacterium</i> , peptostreptococci
Upper respiratory tract	AGPC, <i>Actinomyces</i> , <i>Propionibacterium</i> , <i>Campylobacter</i> , <i>Fusobacterium</i> , <i>Prevotella</i> , <i>Veillonella</i>
Oral cavity	<i>Actinomyces</i> , <i>Eubacterium</i> /Eggerthella, AGPC, <i>Campylobacter</i> , <i>Fusobacterium</i> , <i>Prevotella</i> , <i>Bifidobacterium</i> , <i>Porphyromonas</i> , <i>Veillonella</i>
Intestine	<i>Bifidobacterium</i> , <i>Eubacterium</i> /Eggerthella, <i>Clostridium</i> , AGPC, <i>Bacteroides fragilis</i> group, <i>Parabacteroides</i> , <i>Bilophila</i> , <i>Campylobacter</i> , <i>Fusobacterium</i> , <i>Porphyromonas</i> , <i>Prevotella</i> , <i>Sutterella</i> , <i>Veillonella</i>
Genitourinary tract	AGPC, <i>Bifidobacterium</i> , <i>Fusobacterium</i> , <i>Lactobacillus</i> , <i>Mobiluncus</i> , <i>Prevotella</i> , <i>Veillonella</i>

AGPC, Anaerobic gram-positive cocci.
Modified from Jouseimies, H. R., et al. (2002). *Wadsworth-KTL anaerobic bacteriology manual* (6th ed.). Belmont, CA: Star Publishing.

invasion through the oral mucosa should be suspected whenever oral anaerobes such as *Fusobacterium nucleatum* and *Porphyromonas* are recovered from the bloodstream or from abscesses located far from the oral cavity.

Gastrointestinal tract

It is estimated that 500 to 1000 different species of bacteria live in the GI tract. Microbiota studies have found that anaerobes outnumber facultative anaerobes by a factor of 1000:1. Although *B. fragilis* is the most common species of anaerobic bacteria isolated from soft tissue infections and bacteremia, it accounts for less than 1% of the human intestinal biota. Other members of the *B. fragilis* group, such as *B. vulgatus*, *B. thetaiotaomicron*, and *Parabacteroides distasonis*, are among the most common species of bacteria isolated from human feces. Other species commonly inhabiting the GI tract include species of *Bifidobacterium*, *Clostridium*, *Eubacterium*, and anaerobic gram-positive cocci. Any infection in the peritoneal cavity would likely be caused by organisms that have escaped from the GI tract.

Genitourinary tract

Although anaerobic bacteria colonize the distal urethra, they are not considered to be a cause of uncomplicated urinary tract infections. Similarly, 50% of the bacteria in cervical and vaginal secretions are anaerobes. These include anaerobic cocci, *Fusobacterium*, *Prevotella*, *Bacteroides*, and *Lactobacillus*. For this reason, GU swabs and voided or catheterized urine specimens are unacceptable for anaerobic bacteriology because recovery of these organisms would not distinguish whether they were present as pathogens or as endogenous microbiota.

Factors that predispose patients to anaerobic infections

The precise mechanisms whereby anaerobic bacteria cause disease are not always known. Like other pathogenic bacteria, anaerobes can produce a variety of virulence factors. Toxins, molecules that break down tissue, capsules that inhibit phagocytosis by macrophages, and adherence factors that aid in attachment to mucosal surfaces are thought to play a role in pathogenicity (Table 22.5). Generally, infectious diseases involving anaerobic bacteria follow some type of trauma to protective barriers such as the skin and mucous membranes. Trauma at these sites allows anaerobes of the endogenous biota (or in some cases, soil anaerobes) to gain access to deeper tissues. Any condition causing vascular stasis, decreased blood flow, prevents oxygen from entering a particular site, which results in an environment conducive to growth and multiplication of any anaerobe that might be present at that site. Similar results can occur in the presence of tissue necrosis and when the redox potential in tissue is decreased. Box 22.1 lists examples of conditions that may predispose a patient to anaerobic infections.

Indications of anaerobe involvement in human disease

Infectious processes involving anaerobes are usually purulent, with many polymorphonuclear leukocytes present. However, the absence of leukocytes does not rule out the possibility that anaerobes are contributing to the process because some anaerobes, such as *Clostridium perfringens*, produce enzymatic virulence factors that destroy neutrophils, macrophages, and other cells.

Table 22.5 Potential virulence factors of anaerobic bacteria		
Potential virulence factor	Possible role	Anaerobes known or thought to possess
Polysaccharide capsules	Promotes abscess formation; antiphagocytic function	<i>Bacteroides fragilis</i> , <i>Porphyromonas gingivalis</i>
Adherence factors	Fimbriae, fibrils enable organisms to adhere to cell surfaces	<i>B. fragilis</i> , <i>P. gingivalis</i>
Clostridial toxins, exoenzymes		
Collagenases	Catalyze the degradation of collagen	Certain <i>Clostridium</i> spp.
Cytotoxins	Toxic to specific types of cells	<i>Clostridioides difficile</i>
DNases	Destroy DNA	Certain <i>Clostridium</i> spp.
Enterotoxins	Toxic to cells of the intestinal mucosa	<i>C. difficile</i>
Hemolysins	Lyse red blood cells liberating hemoglobin	Certain <i>Clostridium</i> spp.
Hyaluronidase	Catalyzes the hydrolysis of hyaluronic acid, the cement substance of tissues	Certain <i>Clostridium</i> spp.
Lipases	Catalyze the hydrolysis of ester linkages between fatty acids and glycerol of triglycerides and phospholipids	Certain <i>Clostridium</i> spp.
Neurotoxins (e.g., botulinum toxin, tetanospasmin)	Destroy or disrupt nerve tissue	<i>Clostridium botulinum</i> , <i>Clostridium tetani</i>
Phospholipases	Catalyze the splitting of host phospholipids (lecithinase)	Certain <i>Clostridium</i> spp.
Proteases	Split host proteins by hydrolysis of peptide bonds	Certain <i>Clostridium</i> spp.
DNA, Deoxyribonucleic acid; DNases, deoxyribonucleases.		

BOX 22.1 Conditions that predispose individuals to anaerobe-associated infections or diseases

Vascular disease
 Presence of a foreign body
 Malignancy
 Immune deficiency
 Surgery
 Colitis
 Diabetes mellitus
 Human or animal bite wounds
 Aspiration of oral contents into the lungs after vomiting
 Tooth extraction, oral surgery, or traumatic puncture of the oral cavity
 Gastrointestinal tract surgery or traumatic puncture of the bowel
 Genital tract surgery or traumatic puncture of the genital tract
 Introduction of soil into a wound

BOX 22.2 Indications of involvement of anaerobes in infectious processes

Infection in close proximity to a mucosal surface
 Presence of foul odor
 Presence of large quantity of gas
 Presence of black color (necrosis) or brick red fluorescence
 Presence of "sulfur granules"
 Septic thrombophlebitis
 Bacteremia or endocarditis with no growth on aerobic cultures
 Clinical conditions predisposing to anaerobic infections, see [Box 22.1](#)

[Box 22.2](#) lists indications of anaerobe involvement in infectious processes. Although any of these indicators should alert the physician to the possible involvement of anaerobes, most are not specific for anaerobes. For example, large quantities of gas in the specimen might be caused by gas-producing organisms such as *E. coli* or a mixture of enteric bacteria other than anaerobes. Similarly, the foul odor usually associated with specimens containing anaerobes could be absent. Many of the infectious processes involving anaerobes are polymicrobial, consisting of mixtures of obligate anaerobes or mixtures of obligate or aerotolerant anaerobes and facultative organisms. A symbiotic relationship between facultative anaerobes and obligate anaerobes frequently exists in polymicrobial infections, which can contribute to an infectious disease. In these situations, facultative anaerobic bacteria consume oxygen and reduce the oxygen concentration, thus enhancing the growth of obligate anaerobic bacteria.

Frequently encountered anaerobes and their associated diseases

Taxonomically, anaerobic bacteria encountered in human clinical specimens may be divided into gram-negative and gram-positive genera. Anaerobic gram-positive bacilli can be further divided between those organisms capable of forming endospores (spore formers) and those unable to form spores (non-spore formers). The presence or absence of spores, coupled with the microscopic morphology on Gram-stained smears and colony morphology of culture isolates, can be helpful in making

a presumptive identification of anaerobic bacteria and determining the appropriate identification tests to be performed.

As with other areas of clinical microbiology, the use of molecular methods rather than phenotypic characteristics to determine taxonomic placement of an organism has resulted in an explosion of taxonomic changes. It is beyond the scope of this chapter to list all of the current names of rarely encountered anaerobes. Instead, this discussion will concentrate on the important anaerobes most likely to be encountered in specimens submitted to the clinical microbiology laboratory.

Gram-positive, spore-forming anaerobic bacilli

All spore-forming anaerobic bacilli are classified in the genera *Clostridium* or *Clostridioides* and are collectively termed *clostridia*. Although all clostridia are capable of producing spores, some species do so readily, whereas others require extremely harsh conditions. Spores are often not observed in Gram-stained smears of clinical specimens containing clostridia or in smears of colonies from an agar plate unless the culture has been incubated for many days. It is sometimes necessary to use heat or alcohol shock to induce sporulation.

Clostridia can be grouped according to the location of the endospore within the cell. Spores are described as *terminal* when they are located at the end of the bacterial cell and *subterminal* when the spore is found at a location other than the end of the cell. Terminal spores typically cause swelling of the cell. [Fig. 22.1](#) shows a clostridial species that produces terminal spores and a clostridial species that produces subterminal spores. Spores located in the center of the cell are called *central spores*.

Clinical infections

Clostridium spp. are frequently encountered in exogenous anaerobic infections or intoxications. Clostridia or their toxins usually gain access to the body through ingestion or open wounds that have become contaminated with soil. Clostridia cause classic diseases such as tetanus, gas gangrene (myonecrosis), botulism, and food poisoning (foodborne intoxication). In tetanus, gas gangrene, and wound botulism, clostridial spores enter through open wounds and germinate in vivo. The vegetative bacteria then multiply and produce toxins. In foodborne disease caused by *C. perfringens*, organisms are acquired through the consumption of contaminated food that has been improperly stored. Almost any *Clostridium* spp. can cause wound or abscess infection, usually as part of mixed biota, and most can be isolated from the blood in cases of bacteremia. One clostridial infection of endogenous origin is antibiotic-associated pseudomembranous colitis caused by *C. difficile*.

***Clostridium perfringens* food poisoning**

C. perfringens is associated with two types of food poisoning: type A, a relatively mild and self-limiting GI illness, and type C, a more serious but rarely seen disease. *C. perfringens* foodborne disease usually follows the ingestion of large numbers of enterotoxin-producing strains in contaminated food. *C. perfringens* lacks the ability to produce a number of essential

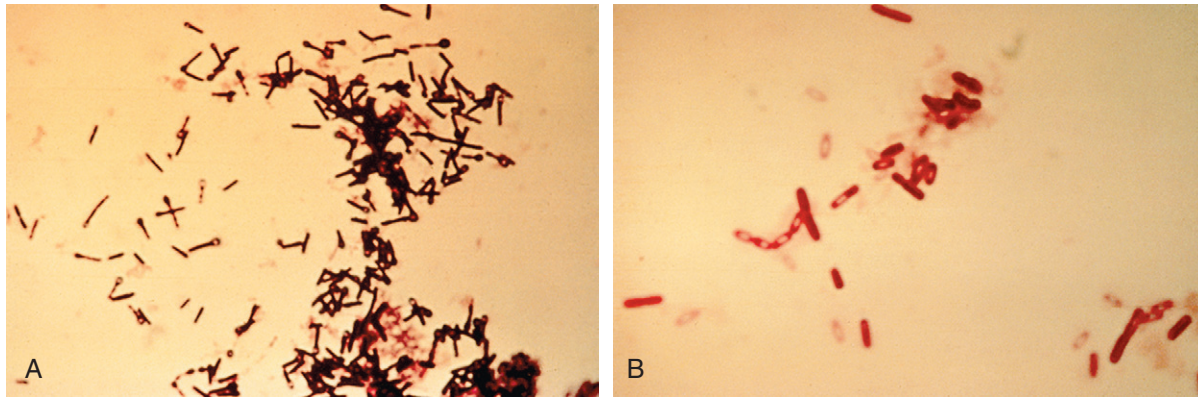


Fig. 22.1 Classification of some clinically encountered clostridia by endospore location. **A**, Gram-stained appearance of terminal spores of *Clostridium tetani* ($\times 1000$). **B**, Gram-stained appearance of subterminal spores of *Clostridium sordellii* ($\times 1000$). (Courtesy S.L. Bartley, J.D. Howard, and R. Simon, Centers for Disease Control and Prevention, Atlanta, GA.)

amino acids, so meats and gravies are commonly implicated in outbreaks. Foods are often heated, which kills vegetative bacteria, but the spore-forming clostridia survive. Improperly stored food allows germination of the spores and growth of vegetative bacteria. Food poisoning caused by *C. perfringens* type A results from an enterotoxin linked to sporulation. After an 8- to 30-hour incubation period, the patient experiences diarrhea and cramping abdominal pain for about 24 hours. Other than fluid replacement, therapy is usually unnecessary.

C. perfringens type C food poisoning (**enteritis necroticans**) is associated with strains that produce β -toxin and, less commonly, α -toxin. After an incubation period of at least 5 to 6 hours, symptoms begin as an acute onset of severe abdominal pain and diarrhea, which is often bloody, and may be accompanied by vomiting. Early symptoms are followed by necrotic inflammation of the small intestines, at times leading to bowel perforation. Without treatment, the disease is often fatal; even with treatment, the fatality rate is 15% to 25%. In general, the clinical microbiology laboratory usually does not have a role in recovering *C. perfringens* from food or feces during outbreaks unless serving a public health function.

Botulism

Foodborne **botulism** results from the ingestion of preformed botulinum toxin, produced in food by *C. botulinum*. Although there are seven antigenically distinct botulinum toxins (A to G), only types A, B, and E are associated with human disease. Botulinum toxin is an extremely potent neurotoxin; only a small amount produces death. Botulinum toxin attaches to the neuromuscular junction and prevents the release of acetylcholine (a neurotransmitter) from nerve cells, which results in flaccid paralysis and death. Botulinum toxin type A (Botox) is also used medically to treat strabismus (crossing eyes) and chronic migraines, and as a beauty enhancer by temporarily improving facial wrinkles.

The food sources involved commonly in botulism include home-canned vegetables, home-cured meat such as ham, fermented fish, and other preserved foods. Clinical manifestations develop as early as 2 hours or as late as 8 days following ingestion of food containing the botulinum toxin. The toxin is absorbed through the small intestine and enters the systemic circulation to reach the nervous system. Weakness and paralysis are the main features of botulism. Double or blurred vision, impaired speech, and difficulty swallowing are also commonly seen. Respiratory paralysis may occur in severe cases. Treatment of foodborne botulism involves the use of

antitoxin and supportive care. Infant botulism, unlike that in adults, follows the ingestion of *C. botulinum* spores. The food most commonly associated with infant botulism is honey contaminated with *C. botulinum* spores. Once ingested, the spores germinate, and the vegetative cells colonize the colon and subsequently produce toxin.

Wound botulism is the result of contamination of wounds with spores of *C. botulinum*, which germinate; the vegetative cells multiply and produce toxins. Clinical manifestations of wound botulism are similar to those of foodborne intoxication. Botulism toxin and *C. botulinum* are considered potential bioterrorism agents; laboratorians or physicians are required to notify public health laboratories if *C. botulinum* is isolated or if a case of botulism is suspected. See [Chapter 30](#) for a discussion of *C. botulinum* as a bioterrorism agent. Many clinical laboratories consider the isolation of *C. botulinum* a critical result, requiring that the nursing unit or physician be notified immediately. In 2018, the Centers for Disease Control and Prevention (CDC) reported 18 cases of foodborne botulism, 162 cases of infant botulism, and 61 cases of wound botulism infections. All cases of wound botulism were reported in individuals who injected drugs.

Tetanus

The clinical manifestations of **tetanus** are attributed to the neurotoxin tetanospasmin produced by *Clostridium tetani*. Tetanospasmin acts on neurons, preventing the release of inhibitory and excitatory neurotransmitters. This results in a spastic type of paralysis, with continuous muscular spasms leading to trismus (lockjaw), risus sardonicus (distorted grin), and difficulty breathing.

Tetanus occurs when spores in the environment enter the skin through puncture wounds. The spores germinate into vegetative cells that produce tetanospasmin. Symptoms usually appear approximately 7 days after the injury, but the incubation period can range from 3 to 21 days. The length of incubation is related to the distance from the injury to the central nervous system. Clinical manifestations include muscular rigidity, usually in the jaw, neck, and lumbar region. Difficulty swallowing results from muscular spasms in the pharyngeal area. Rigidity of the abdomen, chest, back, and limbs may also occur. As a result of the widespread use of the diphtheria-tetanus-acellular pertussis (DTaP) vaccine, tetanus is no longer a common disease in the United States; only 33 cases and 2 deaths were reported to the CDC in 2017. Therapy for tetanus requires injection of antitoxin, muscle relaxants, and

intensive therapy. Although most treated patients completely recover, long-term disability and even death can occur.

Myonecrosis

Myonecrosis, or gas gangrene, usually occurs when organisms contaminate wounds, through trauma or surgery. *C. perfringens*, *C. histolyticum*, *C. septicum*, *C. novyi*, and *C. bifermentans* have all been associated with myonecrosis. *C. perfringens*, however, is the most common cause. Under favorable conditions, the organisms are able to grow, multiply, and release potent exotoxins. In gas gangrene, exotoxins such as α -toxin produced by *C. perfringens* cause necrosis of the tissue and allow deeper penetration by the organisms.

α -Toxin is a lecithinase (phospholipase C) produced by all strains of *C. perfringens*. Clinical manifestations of myonecrosis include pain and swelling in the affected area. Bullae (fluid-filled blisters), serous discharge, discoloration, and tissue necrosis are observed. The onset and spread of myonecrosis can be rapid, and extensive surgical debridement of the necrotic tissue is often required. If treatment is delayed, amputation of the affected limb is not uncommon.

Case check 22.2

The patient's infection in the Case in Point is a typical scenario of gas gangrene (myonecrosis) caused by *C. perfringens*. The tissue exhibited marked necrosis, and gas pockets were noted in the tissue. Rapid surgical debridement is necessary for patient treatment.

Bacteremia

Many of the clostridia have been recovered from blood cultures, but *C. perfringens* is the most common. When *C. septicum* is present in the bloodstream, it is often a marker organism for a malignancy in the GI tract. *C. bifermentans* and *C. tertium* have also been isolated from blood cultures from patients with serious underlying disease.

Clostridioides difficile-associated disease

C. difficile is the most common but not the sole cause of antibiotic-associated diarrhea and **pseudomembranous colitis**, also referred to as ***C. difficile*-associated disease (CDAD)**. This organism is part of the GI biota in about 5% of individuals, although the colonization rate in patients in long-term care facilities, such as nursing homes and rehabilitation facilities, can reach 20% of the population. Following antimicrobial therapy, many bowel biota organisms other than *C. difficile* are killed, thus allowing *C. difficile* to multiply with less competition and produce high levels of two toxins: toxin A, an enterotoxin, and toxin B, a cytotoxin. Bloody diarrhea with associated necrosis of colonic mucosa is seen in patients with pseudomembranous colitis. *C. difficile* is a common cause of healthcare-associated infections. The organism is frequently transmitted among hospitalized patients and is present occasionally on the hands of hospital personnel. Diarrhea caused by *C. difficile* is being seen with increasing frequency in outpatients who have received antimicrobial therapy.

From 2000 to 2015, the incidence of *C. difficile* infections in humans increased in the United States and throughout Europe.

However, the CDC reported a slight decrease in total *C. difficile* infections in 2016 and 2017, with a slight increase in community-acquired cases. In 2017, an estimated 223,900 healthcare-associated *C. difficile* infections and 12,800 deaths occurred. Overall, an estimated 476,000 cases occurred in 2017.

Gram-positive, non-spore-forming anaerobic bacilli

Gram-positive, non-spore-forming anaerobic bacilli have undergone considerable taxonomic revision in recent years. In general, the group can be divided into two phyla, the Actinobacteria and the Firmicutes. Important clinical genera of the Actinobacteria include *Actinomyces*, *Bifidobacterium*, *Eggerthella*, *Mobiluncus*, and *Propionibacterium*. The Firmicutes include many genera, but *Lactobacillus* is the only member encountered on a routine basis in the clinical microbiology laboratory. All of these organisms are found as part of the endogenous microbiota of humans and can be considered opportunistic pathogens. The microscopic morphology of these organisms is varied, ranging from very short rods to long, branching filaments.

Clinical infections

Actinomycosis

Actinomycosis is a chronic, granulomatous, infectious disease characterized by the development of sinus tracts and fistulae, which erupt to the surface and drain pus that may contain so-called *sulfur granules*, dense clumps of bacteria that may be colored. Examinations of wet mounts and Gram-stained preparations of pus from draining sinuses are useful diagnostic procedures for demonstrating the non-spore-forming, thin, gram-positive bacilli that frequently exhibit branching in clinical specimens (Fig. 22.2). Because *Actinomyces* spp. are endogenous biota of the oral cavity, many cases of actinomycosis can be seen in the maxillary region, with draining sinuses in the neck and thorax. Another common site of actinomycosis is the female genital tract, in which the infection is often associated with long standing use of intrauterine devices. Although *Actinomyces israelii* is the most common cause of actinomycosis, other gram-positive anaerobes such

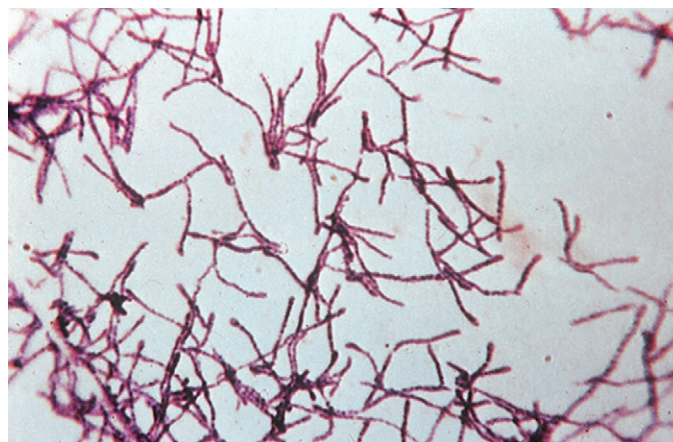


Fig. 22.2 Gram-stained appearance of *Actinomyces israelii*, illustrating the term *Actinomyces*-like ($\times 1000$).

as *Propionibacterium* and *Bifidobacterium* have also been noted to cause this type of infection.

Bacterial vaginosis

Bacterial vaginosis (BV) is thought to arise because of a change in the endogenous microbiota of the vagina. *Lactobacillus* spp. usually constitute the largest portion of the vaginal biota. In BV, a shift in the vaginal biota occurs, resulting in a decrease in the population of lactobacilli and an overgrowth of other endogenous anaerobes of the vagina such as *Mobiluncus* spp., *Bacteroides* spp., *Prevotella* spp., anaerobic gram-positive cocci, and the aerobic bacterium *Gardnerella vaginalis* (see [Chapter 16](#)). Clinical features of BV include a gray-white, homogeneous, malodorous vaginal discharge, with little or no discomfort and no inflammation. BV is associated with an increased risk of acquiring human immunodeficiency virus (HIV) infection and experiencing adverse outcomes in pregnancy and possibly the pathogenesis of pelvic inflammatory disease.

BV is most often diagnosed on clinical appearance and Gram staining of vaginal secretions that reveals the shift in the vaginal biota from predominantly gram-positive lactobacilli to a mixture of *Gardnerella* spp. (gram-variable bacilli) and mixed gram-negative anaerobes. Because many of the anaerobic organisms associated with BV are members of the endogenous microbiota of the female GU tract, culture of vaginal secretions is not performed as part of the diagnosis.

Lactobacillus

Lactobacillus spp. are gram-positive, highly pleomorphic bacilli, which may appear on Gram-stained smears as a coccoid or spiral-shaped organism. There are more than 100 species of *Lactobacillus*. They are considered aerotolerant anaerobes, growing better under anaerobic conditions. Lactobacilli are widely distributed in nature, in foods, and in the normal biota in the human mouth, GI tract, and female genital tract.

Lactobacillus spp. play an important role in the health of the female vaginal tract in that they help protect the host from urogenital infections. Lactobacilli produce lactic acid from glycogen, which lowers the vaginal pH and suppresses the overgrowth of organisms such as *Mobiluncus*, *Prevotella*, and *G. vaginalis*. The *Lactobacillus acidophilus* complex constitutes most of the lactobacilli of the healthy vagina, but *L. fermentum*, *L. vaginalis*, *L. salivarius*, *L. plantarum*, and other species have also been recovered.

Lactobacillus spp. can often be recovered from urine and genital cultures, in which their role in the infectious process is doubtful. Systemic human infections are rare and are associated with the patient's endogenous organisms, typically originating from the oral cavity. Serious infections, primarily bacteremia and endocarditis, are known to occur in immunocompromised patients. Endocarditis is the most common clinical disease caused by lactobacilli and has a high mortality rate (23% to 27%). *Lactobacillus rhamnosus* is the species most often associated with septicemia and endocarditis. Other infections associated with lactobacilli include intraabdominal abscesses, meningitis, oral infections, and conjunctivitis. Opportunistic infections such as endocarditis and polymicrobial abscesses caused by lactobacilli are sometimes seen in patients who previously received the antimicrobial agent vancomycin. Although vancomycin is normally effective in treating infections caused by almost all gram-positive bacteria, many lactobacilli are resistant to the agent and consequently will be able to overgrow other inhibited gram-positive organisms during

vancomycin therapy. In addition to vancomycin, lactobacilli are frequently resistant to the cephalosporins. Once the organism is confirmed as clinically significant and not a contaminant, treatment is usually with penicillin with an aminoglycoside.

Anaerobic gram-negative bacilli

Gram-negative anaerobic bacilli are all non-spore-forming organisms and are often found as members of the endogenous microbiota. They can be found as part of the microbiota of the oral cavity and GI and GU tracts. The bacilli most commonly encountered in clinical specimens include members of the *B. fragilis* group and the genera *Porphyromonas*, *Prevotella*, and *Fusobacterium*.

Clinical infections

Anaerobic gram-negative bacilli are frequently found in mixed infections such as abscesses occurring beneath mucosal surfaces. As predominant members of the GI biota, the organisms are often associated with peritoneal infections following disruption of the GI tract lining. Members of the *B. fragilis* group are the most commonly isolated anaerobes from blood cultures. These agents are more virulent and antimicrobial resistant than many other anaerobic bacteria. *B. fragilis* is the anaerobic species most commonly isolated from blood, ulcers, intraabdominal abscesses, and several other specimens.

Brain abscesses are frequently caused by anaerobic organisms such as *Prevotella*, *Porphyromonas*, and *Fusobacterium* found as endogenous microbiota of the oral cavity. Brain abscesses are often the result of complications from sinusitis, otitis media, and dental infections. The anaerobic gram-negative bacilli are often associated with mixed biota in diabetic foot ulcers and decubitus pressure sores. It is often difficult to determine whether the organisms recovered in culture from these specimens are pathogens or merely colonizers. Lemierre disease, a syndrome of thrombophlebitis of the jugular vein that occurs rarely following group A streptococcal pharyngitis, is caused by *F. necrophorum*. The streptococcal infection produces a peritonsillar abscess containing a number of bacterial species. The abscess aids *F. necrophorum* in penetrating the tissue to reach the jugular veins. *Bilophila wadsworthia* is a human pathogen associated with polymicrobial intraabdominal abscesses and gangrenous appendicitis in children. The bile-resistant *Alistipes* species *A. finegoldii*, *A. onderdonkii*, and *A. shahii* are linked to appendicitis in children and adults and intraabdominal abscesses.

Anaerobic cocci

Most of the anaerobic gram-positive cocci were classified previously in the genus *Peptostreptococcus*, with the exception of *Peptococcus niger*, an infrequent isolate. However, the genus *Peptostreptococcus* has been reclassified into at least six different genera: *Peptostreptococcus*, *Anaerococcus*, *Finegoldia*, *Gallicola*, *Parvimonas*, and *Peptoniphilus*. Taxonomy of this group is still in a state of flux, with new genera being added, so this chapter will continue to use the term *peptostreptococci* or *anaerobic gram-positive cocci* when discussing these organisms as a group.

Although several genera of anaerobic gram-negative anaerobic cocci are found in the endogenous microbiota, only

Veillonella spp. are implicated as pathogens. *Veillonella* are very small (0.3–0.5 µm in diameter) and inhabit the oral cavity. They are usually seen in mixed biota abscesses.

Clinical infections

The anaerobic cocci are weakly pathogenic but can be isolated from a wide variety of infections, including brain abscess, aspiration pneumonia, lung abscess, and gingivitis and other periodontal diseases. They are usually associated with polymicrobial infections but can occasionally be recovered from blood cultures and can cause infections following orthopedic surgery. *Finnegoldia magna* is the most pathogenic of the anaerobic cocci and the one most often isolated in pure culture, including cases of endocarditis, meningitis, and pneumonia. *Peptostreptococcus anaerobius* is linked to polymicrobial abscesses of the brain, ear, jaw, pleural cavity, and other sites. Additional important species include *Peptoniphilus asaccharolyticus*, *Peptoniphilus indolicus*, *Peptoniphilus harei*, *Parvimonas micra*, *Anaerococcus vaginalis*, and *Anaerococcus prevotii*. Evaluating the significance of the anaerobic gram-positive cocci isolated in clinical specimens is often difficult.

Specimen selection, collection, transport, and processing

Specimen quality

When health care providers suspect an infection involving anaerobes, the specimen collected must be from the actual site of the infection and not just a swab of a mucosal surface. It must also be collected in a manner that avoids prolonged exposure to oxygen and must be transported as quickly as possible to the laboratory under anaerobic conditions. The laboratory plays an important preanalytic role in specimen selection by providing guidelines for specimen collection and by ensuring that a suitable anaerobic transport system is available.

As noted, most anaerobic infections are caused by members of the endogenous microbiota. An improperly collected specimen may result in the growth of many different anaerobes, making the interpretation of culture results difficult. The consequences of working up improper or incorrectly collected or transported specimens places a burden on the laboratory and may provide the health care provider misleading results. Consequently, the laboratory, with the cooperation of the medical staff, must develop criteria for the rejection of inappropriate specimens. Specimens likely to contain anaerobic bacteria as normal microbiota are inappropriate for anaerobic culture. Specimens that are acceptable for anaerobic culture are listed in Table 22.6. Conversely, Box 22.3 lists specimens not recommended for anaerobic culture. All of these specimens are likely to result in the growth of many different anaerobes that are colonizing the mucosal surface at the site of specimen collection, making it difficult to determine which, if any, are causing infection.

Specimen transport and processing

Regardless of the type of specimen submitted for anaerobic bacteriology, it must be transported and processed as

Table 22.6 Acceptable specimens for anaerobic bacteriology

Anatomic source	Specimens and recommended methods of collection
Central nervous system	Cerebrospinal fluid, aspirated abscess material, tissue from biopsy or autopsy
Dental, ear-nose-throat specimens	Aspirated abscess material, e.g., nasal sinus; biopsied tissue
Localized abscesses	Needle and syringe aspiration of closed abscesses
Decubitus ulcers	Aspirated pus
Sinus tracts or draining wounds	Aspirated material
Deep tissue or bone	Specimens obtained during surgery from depths of wound or underlying bone lesion
Pulmonary	Aspirate obtained by direct lung puncture, transtracheal aspirate, pleural fluid obtained by thoracentesis, open lung biopsy, sulfur granules from draining fistula
Intraabdominal	Aspirate from abscess, ascites fluid, peritoneal fluid, tissue
Urinary tract	Suprapubic bladder aspiration, cystoscopic urine
Female genital tract	Aspirate from loculated abscess; culdocentesis fluid aspirate, fallopian tube fluid or tissue, IUD for actinomycosis, placenta tissue
Other	Blood, bone marrow, synovial fluid, biopsied tissue from any normally sterile site

IUD, Intrauterine device.

Modified from Engelkirk, P. G., et al. (1992). *Principles and practice of clinical anaerobic bacteriology*. Belmont, CA: Star Publishing.

BOX 22.3 Unacceptable specimens for anaerobic bacteriology^a

- Throat swabs, nasopharyngeal swabs; sputum obtained by nasotracheal or endotracheal suction; bronchial washings or other specimens obtained via a bronchoscope (unless a protected double-lumen catheter is used); expectorated sputum
- Gingival swabs or any other intraoral surface swabs
- Large bowel contents^b; feces^b; ileostomy and colostomy effluents; rectal swabs; gastric and small bowel contents
- Voided or catheterized urine
- Vaginal, cervical, or urethral swabs; female genital tract specimens collected via the vagina^c; swabs of a vaginal discharge
- Surface swabs from decubitus ulcers, perirectal abscesses, foot ulcers, exposed wounds, eschars, pilonidal sinuses, and other sinus tracts
- Any material adjacent to a mucous membrane that has not been adequately decontaminated

^aAll would contain endogenous anaerobic microbiota.

^bExcept for *Clostridioides difficile*, *Clostridium botulinum*, and other specific causative agents.

^cExcept for suction curettages or other specimens collected via a double-lumen catheter.

rapidly as possible and with minimum exposure to oxygen. Specimens are usually collected from a warm, moist environment that is low in oxygen. It is important to avoid shocking the anaerobes by exposing them to oxygen or permitting them to dry out. In addition, the specimens should not be refrigerated, and the amount of time they remain at room temperature should be minimized.

Aspirates

Abscess specimens collected by needle and syringe are better for anaerobic bacteriology than those collected by swab because they are less likely to be contaminated by endogenous microbiota present at the mucosal or skin surface; also, swabs collect less material. The aspirate should be injected into an oxygen-free transport tube or vial containing a prereduced, anaerobically sterilized (PRAS) transport medium, such as the one shown in Fig. 22.3. PRAS media (Anaerobe Systems, Morgan Hill, CA) are prepared by boiling (to remove dissolved oxygen), autoclaving (to sterilize the mixture), and replacing any air with an oxygen-free gas mixture. For serosanguinous fluids that might clot, the anticoagulant EDTA should be used.

Once in the laboratory, aspirates in transport containers should be vortexed to ensure even distribution of the material, especially when the sample is grossly purulent. Using a sterile Pasteur pipette, one drop of purulent material or two to three drops of nonpurulent material should be added to each plate and streaked to obtain well-isolated colonies. A few drops of the specimen may also be inoculated into the bottom of a tube of enriched thioglycollate or cooked meat broth. Finally, one drop of material is spread evenly over an alcohol-cleaned glass slide for Gram staining. The slide should be air-dried and fixed with methanol, not heat-fixed.

Swabs

Swabs, in general, are not appropriate for anaerobic culture. They should be used only when aspiration of material is not possible and a biopsy specimen is not available. When swabs are deemed necessary, they should always be transported under anaerobic conditions. A number of swab transport systems suitable for anaerobes are commercially available.

On arrival in the laboratory, the swab should be placed into a tube containing about 0.5 mL of sterile thioglycollate broth. The swab is vortexed vigorously to remove the clinical material from the swab and then pressed firmly against the inner wall of the tube to remove as much liquid as possible. The remaining liquid suspension is used to inoculate media, as described earlier for an aspirate. Alternatively, the ESwab transport system (COPAN Diagnostics, Murrieta, CA; see Fig. 22.3), which

contains a swab transported in 1 mL of a prereduced liquid Amies medium that can maintain aerobic and anaerobic organisms for up to 48 hours at room temperature can be used.

Tissue

Tissue specimens collected by biopsy or at autopsy from usually sterile sites are acceptable specimens for anaerobic culture. Small pieces of tissue can be placed in anaerobic transport tubes or vials containing PRAS medium to keep the tissue moist. When inserting swabs or small pieces of tissue into an anaerobic transport container, care must be taken not to tip the container. This would cause the heavier than air, oxygen-free gas mixture to be displaced by room air, thus defeating the primary purpose of using such a transport medium.

Larger tissues of more than 1 cm² can maintain a reduced atmosphere as long as the transport time to the laboratory is minimized. These specimens can be sent in a sterile container with sterile, wet gauze. If a delay in transport is expected, tissue may be transported in pouches containing an oxygen-free atmosphere. These bags or pouches are available commercially from Becton-Dickinson Diagnostic Systems (Sparks, MD; GasPak pouches), Mitsubishi Gas Chemical America (New York, NY; AnaeroPack system), and other companies. These containers are described in more detail later (see the “Anaerobic Incubation of Inoculated Media” section). To process the tissue or bone fragments in the laboratory, 1 mL of sterile thioglycollate broth is added to a sterile tissue grinder. The piece of tissue or bone fragment is homogenized until a thick suspension is obtained. Ideally, this procedure is performed in an **anaerobic chamber**. If a chamber is not available, the grinding must be accomplished as quickly as possible in a biological safety cabinet. The suspension is used to inoculate media, as described earlier for an aspirate.

Blood

Blood must be cultured to recover any bacteria or yeasts that might be present. This usually requires aseptic inoculation of anaerobic and aerobic blood culture bottles. At least one anaerobic blood culture bottle should be used. Once inoculated, bottles should be rapidly transported to the laboratory, where they are incubated at 35° to 37° C. Blood for culture must be carefully collected to minimize contamination with skin biota. This usually is accomplished by meticulous preparation of the venipuncture site with a bactericidal agent, such as tincture of iodine, iodophor, or chlorhexidine gluconate in combination with 70% isopropyl alcohol. Blood culture collection is covered in more detail in Chapter 36.

Processing clinical samples for recovery of anaerobic pathogens

To ensure that results of anaerobic cultures are clinically significant, only properly selected, collected, and transported specimens should be processed. Ideally, once a specimen arrives in the laboratory, it should be placed immediately into an anaerobic chamber to prevent further exposure to oxygen. Anaerobic chambers allow all steps in the processing of a specimen to be performed in an oxygen-free environment. In laboratories not equipped with anaerobic chambers, holding

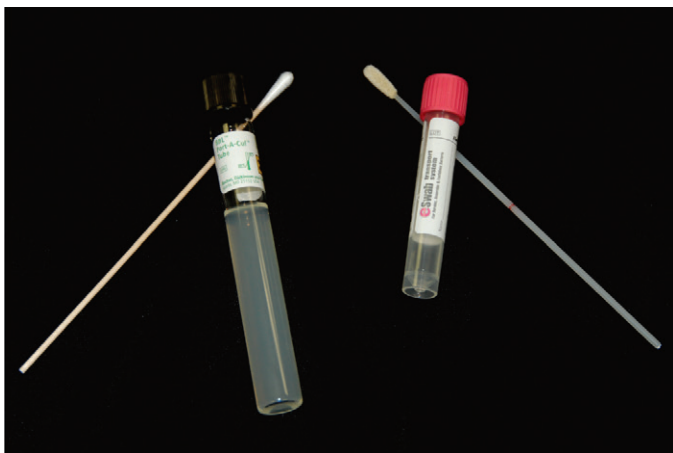


Fig. 22.3 Anaerobic specimen collection and transport systems. *Left*, BBL Port-A-Cul with prereduced gel. *Right*, ESwab with prereduced liquid Amies medium. (BBL Port-A-Cul courtesy BD Diagnostic Systems, Sparks, MD; ESwab courtesy COPAN Diagnostics, Murrieta, CA.)

systems may be used (see the “Inoculation Procedures” section later in this chapter). To comply with mandatory infectious disease safety policies, laboratory scientists must follow appropriate safety precautions. Disposable gloves should be worn and a biosafety cabinet used when handling clinical specimens containing potentially infectious agents. The following procedures should be performed on clinical specimens for the recovery of anaerobic bacteria:

- Macroscopic examination of the specimen
- Preparation of Gram-stained smears for microscopic examination
- Inoculation of appropriately plated and tubed media, including media specifically designed for culturing anaerobes
- Anaerobic incubation of inoculated media

Macroscopic examination of specimens

Each specimen received in the anaerobic bacteriology section should be examined macroscopically and pertinent observations recorded. Some characteristics to note during the macroscopic examination are listed in [Table 22.7](#).

Direct microscopic examination of specimens

Direct smears for Gram stain should be prepared on all specimens received for anaerobic culture for the following reasons:

- The Gram-stained smear reveals the various morphotypes and relative number of microorganisms present in the specimen. The presence of multiple distinct morphologic forms suggests that a polymicrobial infectious process is present.
- Certain morphotypes might provide a presumptive identification of organisms and serve as a guide to media selection. If large, gram-positive bacilli suggestive of clostridia are seen, especially in cases of myonecrosis, the laboratory may want to inoculate an egg yolk agar (EYA) plate to detect lecithinase or lipase activity in addition to the media normally inoculated. Thin,

gram-negative bacilli with tapered ends are suggestive of *Fusobacterium nucleatum*, whereas extremely pleomorphic, gram-negative bacilli with bizarre shapes are suggestive of *Fusobacterium mortiferum* or *F. necrophorum*. Gram-negative coccobacilli may be *Bacteroides*, *Porphyromonas*, or *Prevotella*. The presence of gram-negative coccobacilli with gram-negative fusiforms along with many polymorphonuclear white blood cells in a sputum sample might indicate aspiration pneumonia.

- The Gram-stained smear often reveals the presence of leukocytes, indicating an inflammatory response at the site of the infection. However, certain anaerobes produce necrotizing toxins (leukocidins) that destroy leukocytes. Thus the absence of leukocytes in a Gram-stained smear can never rule out the involvement of anaerobes.
- The Gram-stained smear may also reveal the presence of squamous epithelial cells that would suggest mucosal surface contamination during specimen collection. Such a specimen would likely not provide useful information to the clinician. The laboratory should be cautious in processing such a specimen.
- Finally, the Gram-stained smear can serve as a valuable quality control tool. Failure to isolate certain organisms observed in the direct Gram-stained smear might indicate that problems exist with the anaerobic culture procedure being used. Failure to recover certain morphotypes might be the result of oxygen exposure during specimen collection and transport or because the patient received antimicrobial agents that inhibited growth of the organisms on the plated media.

It is recommended that direct smears for Gram stain be methanol-fixed rather than heat-fixed. Methanol fixation preserves the morphology of leukocytes and bacteria better than heat fixation. Gram-negative anaerobes frequently stain a pale pink when safranin is used as the counterstain and thus may be overlooked in Gram-stained smears of clinical specimens and blood cultures. To enhance the red color of gram-negative anaerobes, the use of 0.1% basic fuchsin as the counterstain or extension of the counterstaining with safranin for 3 to 5 minutes is recommended.

Table 22.7 Characteristics to note during the macroscopic examination of a specimen for anaerobic culture

Questions to ask	Comments
Is it an appropriate specimen?	Inappropriate specimens should be rejected
Was it submitted in an appropriate transport container or medium?	Improperly transported specimens should be rejected
Does the specimen have a foul odor?	Many anaerobes, especially <i>Fusobacterium</i> and <i>Porphyromonas</i> , have foul-smelling metabolic end products
Does the specimen fluoresce brick red when exposed to long-wave (366-nm) ultraviolet light?	Pigmented species of <i>Porphyromonas</i> and <i>Prevotella</i> produce substances that fluoresce under long-wave ultraviolet light before becoming darkly pigmented. Although a brick red fluorescence is presumptive evidence of these organisms, some members of this group fluoresce colors other than brick red
Is the tissue necrotic or exudate black?	Such discoloration may be caused by the pigment produced by pigmented species of <i>Porphyromonas</i> and <i>Prevotella</i>
Does the specimen contain sulfur granules?	Such granules are associated with actinomycosis, a condition caused by <i>Actinomyces</i> , <i>Pseudopropionibacterium propionicum</i> , and closely related organisms, such as <i>Cutibacterium acnes</i> .

Modified from Engelkirk, P. G., et al. (1992). *Principles and practice of clinical anaerobic bacteriology*. Belmont, CA: Star Publishing.

Inoculation of appropriate plated and tubed media

The choice of media for use in the anaerobic bacteriology laboratory is an extremely important aspect of successful anaerobic bacteriology. Anaerobes are fastidious and have special nutritional requirements. Media for obligate anaerobes should contain horse or sheep red blood cells and be supplemented with vitamin K, hemin, and yeast extract. No one medium is likely to support the growth of all anaerobic bacteria; however, CDC blood agar provides the best recovery.

Primary plating media for anaerobic cultures

Table 22.8 lists the primary media recommended for the recovery of anaerobes. Although these media are designed for anaerobes, they also support the growth of most aerobes, so selective media are useful to inhibit facultative anaerobes. At a minimum, specimens for anaerobic culture should be plated onto a nonselective blood agar (e.g., Brucella agar or CDC blood agar), a biplate containing *Bacteroides bile esculin*

(BBE), and kanamycin and vancomycin with laked sheep blood (KVLB) agars. Laked blood is red blood cells lysed through a series of freeze-thaw cycles. Many laboratories also inoculate specimens onto a phenylethyl alcohol (PEA) or colistin–nalidixic acid (CNA) blood agar plate. Either of these media will inhibit facultative anaerobic gram-negative bacteria that can overgrow anaerobes present in the specimen. Surgical specimens or sterile site specimens should also be inoculated into an anaerobic broth medium such as thioglycollate or prereduced chopped meat to serve as a backup source of culture material. If the primary plates do not recover anaerobes, the broth should be subcultured to anaerobic plates. The broth should be held for up to 14 days.

The ideal media for use in the culture of anaerobes are those that have never been exposed to oxygen or have been exposed only briefly. Media should be at room temperature and prereduced when inoculated. Media exposed to air for extended periods may contain toxic substances, produced

Table 22.8 Primary setup media recommended for recovery of anaerobes^a

Medium	Organisms	Comments
Anaerobic blood agar (e.g., CDC medium)	Supports growth of almost all obligate and facultative anaerobes, best for anaerobic gram-positive cocci	An enriched medium containing horse or sheep red blood cells for enrichment and detection of hemolysis, hemin, vitamin K (required by some <i>Porphyromonas</i> spp.), and yeast extract
<i>Bacteroides bile esculin</i> agar	Supports growth of bile-tolerant <i>Bacteroides</i> spp., some strains of <i>Fusobacterium mortiferum</i> , <i>Klebsiella pneumoniae</i> , enterococci, and yeasts may grow to a limited extent	A selective medium containing gentamicin (which inhibits most aerobic organisms), 20% bile (which inhibits most anaerobes), and esculin; used primarily for rapid isolation and presumptive identification of members of the <i>B. fragilis</i> group, which grow well on <i>Bacteroides bile esculin</i> agar (because of their bile tolerance) and turn the originally light-yellow medium to black (because of esculin hydrolysis)
Brucella blood agar	Supports growth of almost all obligate and facultative anaerobes, best for gram-negative bacteria	An enriched medium containing sheep red blood cells for enrichment and detection of hemolysis, casein peptones, dextrose, yeast extract, vitamin K, and hemin
Kanamycin–vancomycin–laked blood agar	Supports growth of <i>Bacteroides</i> and <i>Prevotella</i> spp.; yeasts and kanamycin-resistant, facultative, gram-negative bacilli will also grow	A selective medium containing kanamycin (which inhibits most facultative gram-negative bacilli), vancomycin (which inhibits most gram-positive organisms and vancomycin-sensitive strains of <i>Porphyromonas</i> spp.), and laked blood (which accelerates production of brown-black pigmented colonies by certain <i>Prevotella</i> spp.); used primarily for rapid isolation and presumptive identification of pigmented species of <i>Prevotella</i>
Phenylethyl alcohol agar	Supports growth of almost all obligate anaerobes (gram-positive and gram-negative) and gram-positive, facultative anaerobes	A selective medium containing sheep red blood cells and phenylethyl alcohol; used primarily to suppress the growth of any facultative, gram-negative bacilli (e.g., Enterobacterales) that might be present in the clinical specimen, especially swarming <i>Proteus</i> spp.
Colistin–nalidixic acid blood agar plate	Supports growth of almost all obligate anaerobes (gram-positive and gram-negative) and gram-positive, facultative anaerobes	A selective medium containing sheep red blood cells and the antimicrobials colistin and nalidixic acid; used primarily to suppress the growth of any facultative, gram-negative bacilli (e.g., Enterobacterales) that might be present in the clinical specimen, especially swarming <i>Proteus</i> spp.
Anaerobic broth (e.g., thioglycollate and chopped or cooked meat)	Supports growth of almost all types of bacteria; in thioglycollate broth, obligate aerobes and microaerophiles grow near the top, obligate anaerobes at the bottom, and facultative anaerobes throughout the broth	Because obligate anaerobes can be overgrown by more rapidly growing facultative organisms present in the specimen and killed by their toxic, metabolic by-products, thioglycollate broth serves only as a backup source of culture material (e.g., in case there is no growth on plated media because of a jar failure or presence of antimicrobial agents in the specimen); broth cultures should never be relied on exclusively for isolating anaerobes from clinical material

^aNot all media listed are needed for every culture. Each laboratory needs to decide which media are appropriate depending on specimen source and pathogens expected. At a minimum, each culture should include an enriched anaerobic blood agar plate, *Bacteroides bile esculin* agar plate, kanamycin–vancomycin–laked blood agar plate, and an anaerobic broth.

CDC, Centers for Disease Control and Prevention.

Modified from Engelkirk, P. G., et al. (1992). *Principles and practice of clinical anaerobic bacteriology*. Belmont, CA: Star Publishing.



Fig. 22.4 Prereduced, anaerobically sterilized (PRAS) plated media. PRAS plated media are manufactured, packaged, shipped, and stored under anaerobic conditions. (Courtesy Anaerobe Systems, Morgan Hill, CA.)

as a result of the reduction of molecular oxygen. Such media may also have redox potentials above those required for anaerobes to grow. To provide the best recovery of anaerobes, fresh plated media should be stored in an anaerobic chamber or holding system until used. An alternative is to use commercial media, such as PRAS media, that have been prepared, packaged, shipped, and stored under anaerobic conditions. With the PRAS media shown in Fig. 22.4, growth is initiated quickly. Many anaerobes produce sufficient growth after only 24 hours of incubation. Studies have demonstrated that PRAS media perform better than other commercially available media.

Media for aerobic incubation

In most cases, a request for anaerobic culture on a specimen is also accompanied by a request for aerobic culture. In addition to the battery of selective anaerobic media to be incubated anaerobically, a variety of plated media to be incubated in room air and in a CO₂ incubator are also inoculated. The specific media differ somewhat among laboratories and will depend on the specimen type; nevertheless, 5% sheep blood, MacConkey, and chocolate agar plates are usually included. Some laboratories also routinely include a CNA blood agar plate to select for gram-positive bacteria. Fig. 22.5 shows a typical plating protocol for a wound sample and the types of results that might be expected.

Inoculation procedures

In laboratories not equipped with an anaerobic chamber, inoculation of appropriate plated and tubed media could be performed in an area with a suitable nitrogen gas holding system. Such a holding system, which may use a jar, box, or other small chamber, allows plates that have not been inoculated to be held under anaerobic conditions until needed. Inoculated plates should be held under near-anaerobic conditions until placed into an anaerobic jar or bag. Care should be taken to ensure that inoculated plates do not remain in the holding jar at room temperature for extended periods (not

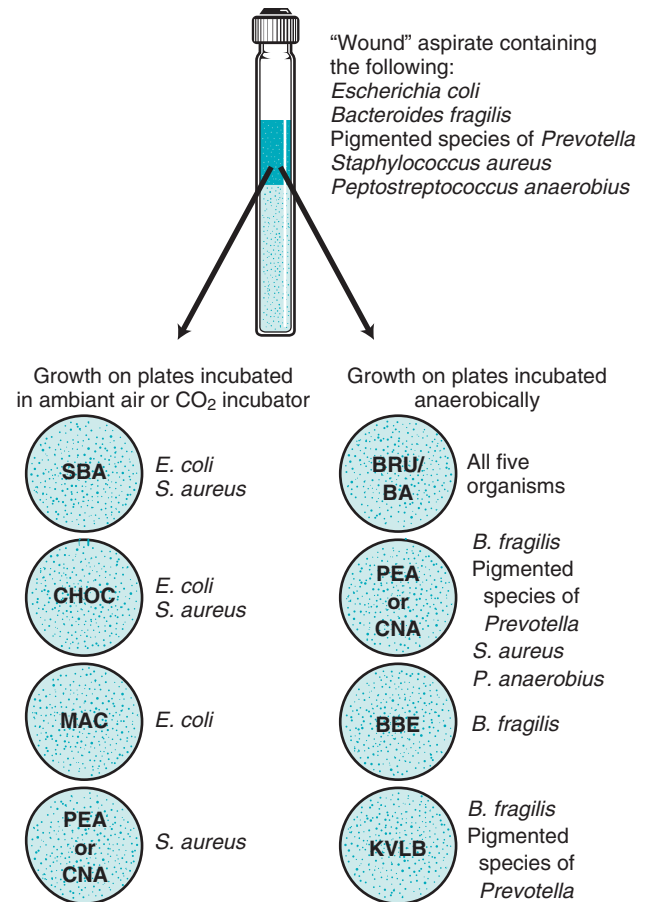


Fig. 22.5 Culture results that might be obtained from the primary isolation of a hypothetical wound specimen. Shown are the media and atmospheric conditions that would support the growth of various organisms contained in the specimen. BBE, Bacteroides bile esculin agar; BRU/BA, Brucella blood agar; CHOC, chocolate agar; CNA, colistin–nalidixic acid blood agar; KVLB, kanamycin–vancomycin–laked blood agar; MAC, MacConkey agar; PEA, phenylethyl alcohol blood agar; SBA, sheep blood agar. (From Engelkirk, P. G., et al. [1992]. *Principles and practice of clinical anaerobic bacteriology*. Belmont, CA: Star Publishing.)

longer than 1 hour). Also, so that the holding jar can maintain as low an oxygen concentration as possible, care should be taken to minimize convection currents whenever freshly inoculated plates are added to the jar. A good quality control procedure to ensure that the agar is maintained under proper conditions is to inoculate an agar plate with *F. nucleatum* and place the plate in the holding jar. After all of the specimens have been inoculated, the agar plate can be incubated anaerobically. Growth of *F. nucleatum* indicates that the agar was maintained under near-anaerobic conditions.

Some microbiologists believe that it is better to batch-process specimens than to process each specimen as it arrives in the laboratory. This relieves the concern that freshly inoculated plates will remain at room temperature in a holding system that may contain some oxygen. Batch processing is an acceptable alternative for aspirates and specimens received in proper transport containers—those kept moist under anaerobic conditions. It would be unacceptable, however, to batch-process improperly submitted specimens (e.g., dry swabs) or clinical specimens apt to contain rapidly growing bacteria. The delay would further expose anaerobes to

molecular oxygen, increase the likelihood of specimens drying out, and decrease the probability of recovering anaerobes.

Anaerobic incubation of inoculated media

After specimens have been processed and inoculated onto the appropriate media, the inoculated plates must be incubated anaerobically at 35° to 37° C. The most common and practical choices for anaerobic incubation systems for clinical laboratories are anaerobic chambers, anaerobic jars, and anaerobic bags or pouches. The choice of system is influenced by a number of factors, including financial considerations, the number of anaerobic cultures performed, and space limitations.

An alternative method is the OxyPlate (Oxyrase, Mansfield, OH), which contains the proprietary substance Oxyrase. This reducing agent protects obligate anaerobes growing on the agar surface. After inoculation, the plate is put into an OxyDish, producing a self-contained environment for growing anaerobes in an ambient air incubator. This system eliminates the use of anaerobic chambers and gas packs.

Anaerobic chambers

The ideal anaerobic incubation system is an anaerobic chamber, which provides an oxygen-free environment for inoculating media and incubating cultures. Identification and susceptibility tests also can be performed in the chamber. Anaerobic chambers are available as sealed glove boxes and gloveless chambers. Glove boxes are fitted with airtight rubber gloves (Fig. 22.6). They are easier to use and less susceptible to oxygen contamination than gloveless chambers. Glove boxes also use less gas and are therefore cheaper to operate. The microbiologist inserts his or her hands into the gloves and manipulates specimens, plates, and tubes inside the chamber. However, they have the disadvantage of trying to accommodate many different hand sizes into one pair of gloves. This disadvantage can be overcome by the use of the gloveless anaerobic chamber (Fig. 22.7). Airtight rubber sleeves that fit snugly against the user's bare forearms are used in place of gloves, enabling the microbiologist to work within

an anaerobic environment with bare hands. However, the cuffs for the arms might not accommodate everyone, allowing air to leak around the arms of smaller individuals. It is recommended that disposable gloves be worn if clinical specimens are being processed in gloveless chambers. Subsequent manipulation of cultures may be performed with bare hands, if preferred. Some gloveless models also have a dissecting microscope mounted on the front of the rigid Plexiglas chamber. This enables the user to observe colony morphology within the chamber, eliminating the need to remove the plates from the chamber, which would result in exposure of the colonies to oxygen.

Anaerobic chambers contain a catalyst, a desiccant, anaerobic gas (5% H₂, 5% to 10% CO₂, and 85% to 90% nitrogen), and an oxidation-reduction indicator. The catalyst, usually palladium-coated alumina pellets, removes residual oxygen from the atmosphere in the chamber. With time, the catalyst pellets become inactivated by water and gaseous metabolic end products produced by the anaerobes, particularly hydrogen sulfide (H₂S). This by-product of metabolism not only "poisons" the catalyst but can harm the chamber's electronics. A product called Anotox (Anaerobe Systems, Morgan Hill, CA) has been shown to absorb these metabolites and prolong catalyst life.

Silica gel is sometimes used as a desiccant to absorb the water formed when H₂ combines with free oxygen in the presence of the catalyst and from water vapor from the bacterial media. Unfortunately, the gel can be overwhelmed and become saturated without the users knowing. Desiccants that change color when saturated are not recommended because of the fine powder they contain that can circulate throughout the chamber. A better, albeit more expensive, method is to use a solid-state dehumidifier.

Carbon dioxide is required for the growth of many anaerobic organisms, and inert nitrogen gas is used as filler for the remaining percentage of the anaerobic atmosphere.



Fig. 22.6 Glove box type of anaerobic chamber. The flexible, clear vinyl chambers are available in three lengths—36, 59, and 78 inches—including one fitted with two pairs of gloves so two microbiologists can use the chamber simultaneously. (Courtesy Coy Laboratory Products, Grass Lake, MI.)



Fig. 22.7 Gloveless type of anaerobic chamber with dissecting microscope attachment. This stainless steel and Plexiglas chamber is manufactured by Anaerobe Systems. (Courtesy Anaerobe Systems, Morgan Hill, CA; Sheldon Manufacturing, Cornelius, OR.)

Anaerobic gas is one of the biggest expenditures in operating an anaerobic chamber. Using high-quality anaerobic gas (calibrated or certified) substantially increases the cost. Most anaerobe chambers can operate with lower-quality gas. Small amounts of oxygen in these gases can generally be removed by the anaerobic chamber.

The College of American Pathologists requires that laboratories performing anaerobic cultures verify daily that anaerobiosis is achieved. This requires the use of an oxygen reduction indicator, which can be methylene blue or resazurin. Methylene blue remains white in the absence of oxygen (reduced) and turns blue in the presence of oxygen; resazurin goes from colorless in the absence of oxygen to pink in the presence of oxygen. These agents can be purchased as strips inside foil pouches and opened inside the chamber or ampules that can be broken to expose the reagent to air just before anaerobic inoculation. A problem with this method is that the agents change color at too high a level of oxygen concentration, which can make it difficult to react in time to maintain the viability of strict anaerobes. Digital oxygen indicators are available that offer faster response times to changes in atmospheric concentrations.

Anaerobic jars

For small laboratories, in which the volume of anaerobic cultures may not justify the purchase of anaerobic chambers, alternative systems are available. One option is the GasPak jar (BD Diagnostic Systems; Fig. 22.8). These jars have been used in clinical laboratories for many years, enabling even small laboratories to perform satisfactory anaerobic bacteriology. Some models accommodate a larger number of plates and microtiter susceptibility trays and anaerobic identification strips or trays. Other anaerobic jars or containers are available from other companies. None of these systems provide all the features or advantages of anaerobic chambers, and cost analysis reveals that over time, a chamber is actually more cost-effective.

Anaerobic jar systems use an envelope gas generator. Numerous gas-generating systems are available and can be divided into newer systems that are waterless and older systems that require water. When water is added to the GasPak



Fig. 22.8 Anaerobic jars. This photograph depicts two of the many different types of anaerobic jars available commercially. As can be seen, the jars can also be used to culture microaerophilic organisms (CampyPak Plus). (Courtesy BD Diagnostic Systems, Sparks, MD.)

envelope, two gases are generated, CO_2 and H_2 . The two gases have a function similar to that in the anaerobic chamber, with H_2 combining with oxygen to form water. Hydrogen is explosive, and if the catalyst is not functioning properly, hydrogen gas will accumulate in the jar. An individual should never open the jar in the vicinity of an open flame. A methylene blue oxidation-reduction indicator strip is always added to the jar to verify that an anaerobic atmosphere was achieved. It takes about 30 to 45 minutes to obtain an anaerobic environment. However, it may take several hours for the methylene blue indicator to change from blue to white. If the catalyst performs properly, water vapor will be present on the inside of the jar, and the indicator strip will be white. Some of the newer gas-generating packets contain a built-in indicator.

Failure to achieve anaerobic conditions could be the result of a “poisoned catalyst” or a crack in the jar, lid, or O-ring. A poisoned catalyst results from the gases, particularly H_2S , produced by anaerobes. The reusable catalyst pellets can be rejuvenated after every use by heating in a 160°C oven for a minimum of 2 hours. The waterless, gas-generating systems (AnaeroPack system, Mitsubishi Gas Chemical America, New York, NY; BD GasPak EZ anaerobe pouch system, BD Diagnostics) are single-use disposable packets designed to produce anaerobic conditions without the use of water or a catalyst. Once the packet has been removed from its foil container, it must be rapidly (within 1 minute) placed in the anaerobe jar or bag. The atmospheric oxygen in the container is quickly absorbed, with the simultaneous generation of CO_2 . The reaction proceeds without the generation of H_2 , so it eliminates the need for a catalyst. No water is produced in response to the anaerobic atmosphere generation, so plates in bags can be more easily viewed. Another alternative is the Anoxomat (Advanced Instruments, Norwood, MA), which is a gas evacuation system that creates an anaerobic environment within 3 minutes (Fig. 22.9).



Fig. 22.9 Advanced Anoxomat Mark II system (Advanced Instruments, Norwood, MA) is a gas evacuation replacement system. (Courtesy Dr. Steven Dallas, University of Texas Health Science Center, San Antonio, TX.)

The major disadvantage of any anaerobic jar system is that the plates must be removed from the jar to be examined and processed. This exposes the bacteria to oxygen, which is especially harmful to the anaerobes during their first 48 hours of growth. For this reason, a suitable holding system should ideally be used in conjunction with anaerobic jars. Plates can be removed from the anaerobic jar, placed in an oxygen-free holding system, removed one by one for rapid examination of colonies, and then quickly returned to the holding system. Plates never should remain in room air on the open bench.

Anaerobic bags

An alternative to an anaerobic chamber or jars are anaerobic bags or pouches (Fig. 22.10). These are available from BD Diagnostic Systems, Mitsubishi, Oxoid (Basingstoke, United Kingdom), Hardy Diagnostics (Santa Monica, CA), and other



Fig. 22.10 Anaerobic pouch. This photograph depicts the GasPak pouch, one of the commercially available anaerobic bag or pouch systems. Identification and susceptibility testing systems requiring anaerobic incubation can be incubated in some bags or pouches. (Courtesy BD Diagnostic Systems, Sparks, MD.)

manufacturers. One or two inoculated plates are placed into a bag, an oxygen removal system is activated, and the bag is sealed and incubated. Theoretically, the plates can be examined for growth without removal of the plates from the bags, so the colonies are not exposed to oxygen. However, with some of the products, a water vapor film on the inner surface of the bag or the lid of the plate can sometimes obscure vision. In such cases, the plates must be removed from the bag to observe them for growth, and a new bag and oxygen removal system must be used whenever additional incubation is required. As with the anaerobic jar, plates must be removed from the bags to work with the colonies at the bench. An anaerobic holding system should therefore be used in conjunction with any of the anaerobic bags.

Any of these bags are also useful transport devices. For example, a biopsy specimen can first be placed into a sterile screw-capped tube containing sterile saline, which is then placed in one of these bags. Once the oxygen removal system has been activated, the specimen is transported to the laboratory in the bag, thereby minimizing exposure to oxygen. This is especially important if delays in transport of the specimen are expected.

Procedures for identifying anaerobic isolates

Various methods are available to clinical microbiologists for identifying anaerobic isolates (Table 22.9). The method and level of identification usually depend on the size and capabilities of the laboratory and whether the organism is from a mixed infection or from a sterile site such as blood or another sterile body fluid (e.g., synovial, peritoneal, or pleural fluid). Presumptive identifications of microorganisms have become more popular in recent years primarily because of increased emphasis on speed and cost reduction. Several anaerobic bacteria can be identified in the laboratory using presumptive identification criteria, thereby providing physicians with timely information concerning the presence of anaerobes in

Table 22.9 Options available for identifying anaerobic bacteria		
Identification technique	Time to obtain results	Extent of identification
Presumptive identification—based on colony morphology, Gram stain observations, and results of simple tests (e.g., disks, catalase, spot indole)	Same day that PC-SC plate is available	Limited capability; many clinically encountered anaerobes cannot be identified
Definitive identification—commercially available, preexisting, enzyme-based identification systems (e.g., ANIDENT, RapID ANA-II, BBL Crystal, Vitek ANI card)	Same day that PC-SC plate is available (4-hour incubation)	Most commonly encountered, clinically significant anaerobes can be identified
Commercially available biochemical-based identification systems (e.g., API 20A, Minitek)	24–48 hours after PC-SC plate is available	Most commonly encountered saccharolytic anaerobes can be identified, but many asaccharolytic anaerobes cannot be identified
Cellular fatty acid analysis by high-resolution GLC (e.g., MIDI system)	24–48 hours after PC-SC plate is available	Most clinically encountered anaerobes can be identified
16S rDNA sequencing	5 hours after PC-SC plate is available	Most clinically encountered anaerobes can be identified
MALDI-TOF mass spectrometry	Less than 1 hour	Most clinically encountered anaerobes can be identified
GLC, Gas-liquid chromatography; MALDI-TOF, matrix-assisted laser desorption/ionization–time-of-flight; PC-SC, pure culture–subculture; rDNA, ribosomal DNA.		

clinical specimens. The following sections on identification techniques are based on progressively more complex procedures that ultimately can provide the genus and species of an anaerobic bacterium. However, often the most actionable information for the health care provider can be based on the presumptive identification procedures described here, and full identification might not be necessary. Communication with the health care provider is important to determine how far to go with an identification and whether susceptibility testing is required for proper patient management.

The Clinical and Laboratory Standards Institute (CLSI) document (M35-A2, *Abbreviated Identification of Bacteria and Yeast*) provides guidelines for the rapid presumptive identification of commonly encountered anaerobic bacteria. It is an individual laboratory's decision whether such identification will suffice or whether definitive identifications of some isolates are necessary. By using the guidelines in the CLSI document, an isolate can be identified with more than 95% accuracy. Consequently, by combining readily observable colony and Gram stain features with results of simple test procedures, even small laboratories can make presumptive identification of commonly isolated and clinically important anaerobes.

Preliminary procedures

When to examine primary plates

Because many anaerobic infections consist of a mixture of aerobic and anaerobic organisms, the most actionable information that can be provided to the physician is a rapid answer about whether or not the specimen contains anaerobes. The health care provider can then select the most appropriate empiric antimicrobial agents to treat the infection.

Plates incubated in an anaerobic chamber can be examined at any time without exposing the colonies to oxygen. However, those incubated in anaerobic jars, bags, or pouches will be exposed to the potentially damaging effects of oxygen whenever the containers are opened. This may inhibit the growth of strict anaerobes because of the toxic effect of oxygen. If a holding system is available at the anaerobe workstation and is used in conjunction with jars, bags, or pouches, exposure to oxygen will be minimized, and plates can be removed and examined at 24 hours and then placed immediately into the holding system. As soon as all plates have been examined, those requiring additional incubation are returned to an anaerobic system and incubated for an appropriate period before reexamination. If a holding system is not used, inoculated plates should be held until 48 hours before initial examination. Because this delays the time to detection, it certainly is not the optimal method; however, it might be necessary in small laboratories lacking resources. Anaerobic cultures are routinely held for 5 to 7 days to allow growth of particularly slow-growing anaerobes and at least 10 days whenever *Actinomyces* is suspected.

Indications of the presence of anaerobes in cultures

Several clues can alert the clinical microbiologist that anaerobes may be present on the primary plates. These include:

- A foul odor on opening an anaerobic jar or bag. Some anaerobes, especially *C. difficile*, *Fusobacterium*, and *Porphyromonas*, produce foul-smelling metabolic end products that are readily apparent when the jars, bags, or pouches are opened.
- Colonies present on the anaerobically incubated blood agar plates but not on the CO₂-incubated blood or chocolate agar plates
- Good growth (>1 mm in diameter) of gray colonies on a BBE agar plate, characteristic of members of the *B. fragilis* group
- Colonies on KVLB agar or anaerobic blood agar plates that fluoresce brick red under ultraviolet (UV) light or are brown to black in ordinary light, characteristics of pigmented *Porphyromonas* and *Prevotella*. However, most strains of *Porphyromonas* will not grow on KVLB agar because of their sensitivity to vancomycin.
- Double zone of hemolysis on SBA plate incubated anaerobically, suggestive of *C. perfringens*

When anaerobes are suspected, the following steps must be performed and recorded for each colony morphotype present on the anaerobic blood agar plate to initiate presumptive identification of the isolates:

- Describe the colony morphology, and note whether growth occurred on each of the selective (e.g., BBE agar, KVLB agar) and nonselective anaerobic blood agar plates.
- Describe the Gram stain reaction and cell morphology.
- Set up an aerotolerance test (see the "Aerotolerance Testing" section).
- Inoculate a pure culture–subculture plate and add appropriate identification disks.

Colony morphology and Gram stain reaction

The use of a dissecting microscope to observe the fine details of each colony morphotype and pick colonies for isolation is recommended. Fig. 22.11 depicts the appearance of colonies as seen through a dissecting microscope. Growth of a

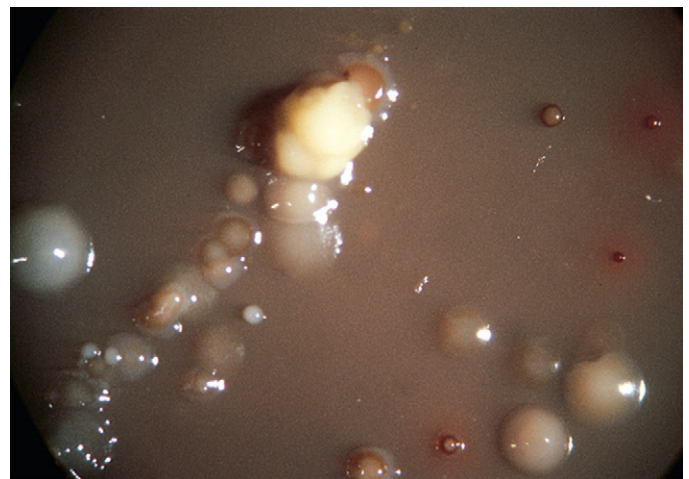


Fig. 22.11 Anaerobic blood agar plate from an intrauterine device culture as seen through a dissecting microscope. The heavy mixture of sizes and types of colonies makes the task of isolating and identifying colonies very difficult without the enhancement obtained with a dissecting microscope.

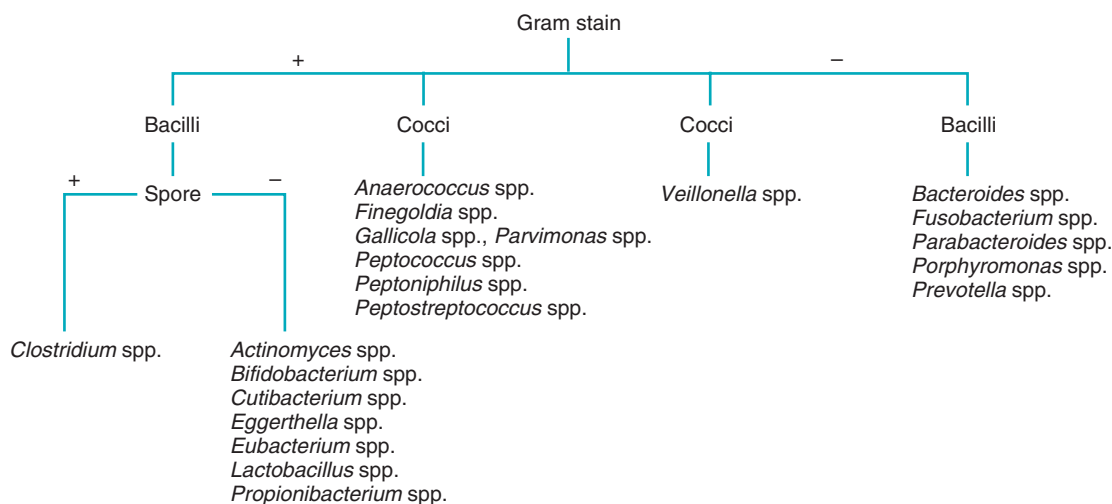


Fig. 22.12 Schematic diagram for the initial identification of anaerobic isolates based on Gram-stain morphology. Not all *Clostridium* spp. readily sporulate in clinical specimens or in culture.

particular morphotype can be semiquantitated using terms such as *light*, *moderate*, and *heavy* or a coding system (e.g., 1+, 2+, 3+). The Gram stain reaction and morphologic appearance of the organism aid in the presumptive identification of isolates, as shown in the algorithm in Fig. 22.12.

Aerotolerance testing

The **aerotolerance test** determines whether a microorganism isolated under anaerobic conditions is a strict or facultative anaerobe. Incubating the suspected isolate in aerobic and anaerobic environments determines the atmospheric requirements of the organism. Suspected colonies should be inoculated with a short streak onto a chocolate agar plate for incubation in a CO₂ incubator and an anaerobic blood agar SBA plate incubated anaerobically. By using this technique, many different organisms can be tested for aerotolerance on a single plate. Some laboratories also prefer to inoculate an SBA plate in the same manner to be incubated in an ambient air incubator. This enables differentiation between aerobic and capnophilic organisms.

All aerotolerance test plates should be incubated at 35° C for up to 48 hours. After incubation, the aerotolerance test plates are examined for growth. An obligate anaerobe would grow only on the anaerobically incubated plates. However, some aerotolerant anaerobes (e.g., certain *Clostridium*, *Actinomyces*, *Propionibacterium*, and *Lactobacillus* spp.) can grow on the CO₂-incubated chocolate agar plate, but they usually grow much better on the anaerobically incubated plate. A facultative, non-capnophilic organism will grow on all plates, but a capnophilic aerobe should grow only on the CO₂-incubated chocolate agar plate. *Haemophilus influenzae*, however, will grow on the anaerobic plate and the CO₂-incubated chocolate agar plate but not on the aerobically incubated SBA plate. Table 22.10 describes how the aerotolerance test results are interpreted.

Presumptive identification of clinically significant anaerobes

A presumptive identification of a bacterium is derived from simple colony and Gram stain observations and the results

Table 22.10 Interpretation of aerotolerance test results

	Obligate aerobe	Capnophilic aerobe	Facultative anaerobe	Obligate anaerobe
Blood agar plate incubated aerobically in a non-CO ₂ incubator	+	–	+	–
Chocolate agar plate incubated aerobically in a CO ₂ incubator	+	+	+	–
Blood agar plate incubated anaerobically	–	– ^a	+	+

^a*Haemophilus influenzae* will grow on an anaerobically incubated Brucella blood agar plate but can be differentiated from an anaerobe by its growth on a chocolate agar plate incubated in a CO₂ incubator.

of several relatively rapid and inexpensive tests. The aerotolerance test must be one of the first tests performed. The next section describes tests, some rapid (results in about 1 hour or less), that are of value in presumptively identifying anaerobes commonly encountered in clinical specimens.

Rapid tests

Fluorescence

Many strains of pigmented *Porphyromonas* and *Prevotella* fluoresce brick red under long-wave (366-nm) UV light (Fig. 22.13A). However, some strains fluoresce colors other than brick red (e.g., brilliant red, yellow, orange, pink-orange).

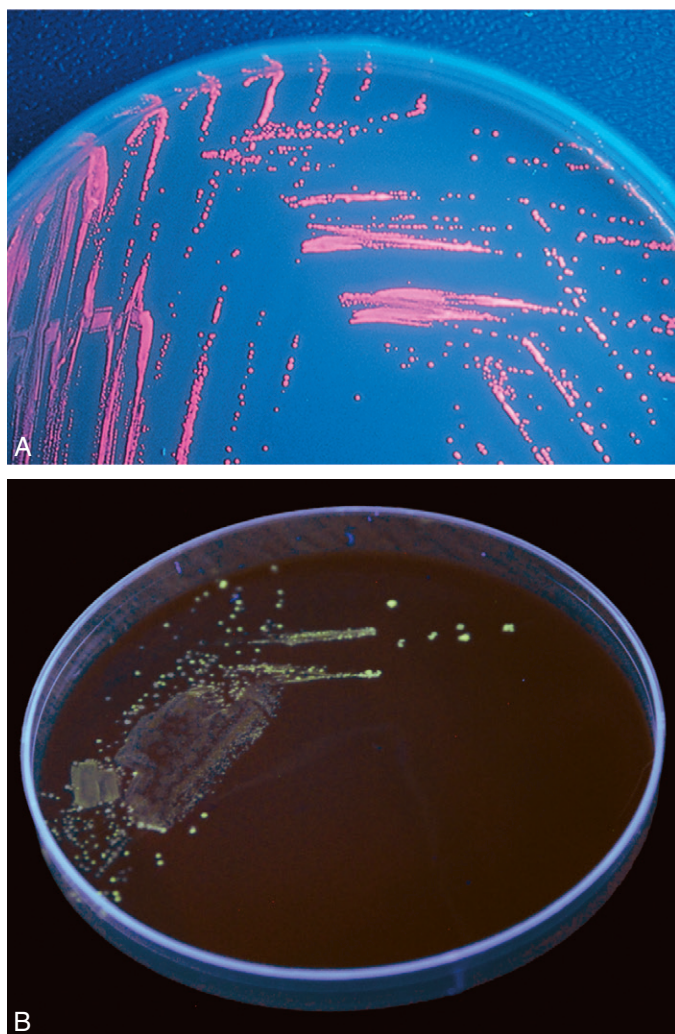


Fig. 22.13 Examples of fluorescence observed with long-wave ultraviolet light. **A**, Brick red fluorescence observed with *Porphyromonas asaccharolytica*. **B**, Chartreuse fluorescence of *Fusobacterium nucleatum*. (A, From Engelkirk P. G., et al. (1992). *Principles and practice of clinical anaerobic bacteriology*. Belmont, CA: Star Publishing.)

F. nucleatum and *C. difficile* fluoresce chartreuse (see Fig. 22.13B). *Veillonella* spp. often fluoresce red, but the fluorescence is dependent on the culture medium. It is weaker than the fluorescence produced by pigmented species of *Porphyromonas* and *Prevotella* and fades completely if the colonies are exposed to air for 5 to 10 minutes. Table 22.11 lists the fluorescence characteristics of commonly isolated anaerobic bacteria.

Catalase test

To perform a catalase test, a plastic, disposable inoculating loop or wooden applicator stick is used to place some of the colony onto a small area of a glass microscope slide. A drop of 15% hydrogen peroxide is added; the production of bubbles of oxygen gas is a positive result. Among other uses, the catalase test is valuable in differentiating aerotolerant strains of *Clostridium* (catalase negative) from *Bacillus* (catalase positive). In some anaerobic species, catalase is an inducible enzyme. The bacteria should be exposed to room air for about 20 minutes before testing.

Table 22.11 Fluorescence under long-wave ultraviolet light

Organism	Color of fluorescence
<i>Prevotella</i> (pigmented)	Brick red
<i>P. bivia</i> , <i>P. disiens</i>	Light orange to pink (coral)
<i>Porphyromonas</i>	Brick red (some no fluorescence)
<i>Fusobacterium</i>	Chartreuse (yellow/green)
<i>Veillonella</i>	Red but fades rapidly
<i>Clostridium ramosum</i>	Red
<i>C. innocuum</i> , <i>C. difficile</i>	Chartreuse
<i>Eggerthella lenta</i>	Red or no fluorescence

Spot indole test

The spot indole test uses a small piece of filter paper saturated with *p*-dimethylamino cinnamaldehyde. An inoculating loop or wooden applicator stick is used to transfer some growth from the culture plate onto the saturated filter paper. Rapid development of a blue or green color indicates a positive test result (production of indole from the amino acid tryptophan), whereas a pink or orange color indicates a negative test result. The spot indole test also can be performed directly on a pure culture plate. The spot indole test is useful in identifying *C. acnes* (indole positive) from the similar *Propionibacterium* spp. (indole negative).

Urease test

A rapid urease test can be accomplished by adding a Wee-Tab urease test tablet (Key Scientific Products, Stamford, TX) to 1 mL of distilled water. The isolate is then heavily inoculated into the water and incubated at 37° C. A positive reaction can occur within 15 minutes, but the tube must be held for up to 6 hours before a true negative reaction is determined. Rapid urea broths are also available from various manufacturers. *C. sordellii* is the only *Clostridium* species that is urease positive.

Motility test

Motility may be determined by wet mount using either very young (4–6 hours old) broth cultures or 24- to 48-hour-old colonies on agar. Using a wooden applicator stick, a small portion of a colony should be touched to a drop of saline on a microscope slide. A coverslip is placed over the drop, and the slide is examined with a light microscope. A positive motility test result is indicated when organisms exhibit purposeful movement, which should not be confused with Brownian motion. Motile gram-negative anaerobes include some *Campylobacter* spp. (e.g., *Campylobacter concisus*, *C. curvus*, and *C. rectus*) and *Mobiluncus* spp., among many others.

Special potency antimicrobial disks

Although the Gram stain is helpful in the initial identification of an anaerobic isolate, certain *Clostridium* spp. can stain pink and thus appear to be gram-negative bacilli. To determine the true Gram stain reaction of the isolate, special potency disks can be used. These disks—kanamycin (1000 µg), vancomycin (5 µg), and colistin (10 µg)—are used for identification purposes and are not meant to predict treatment options for the physician (Fig. 22.14).

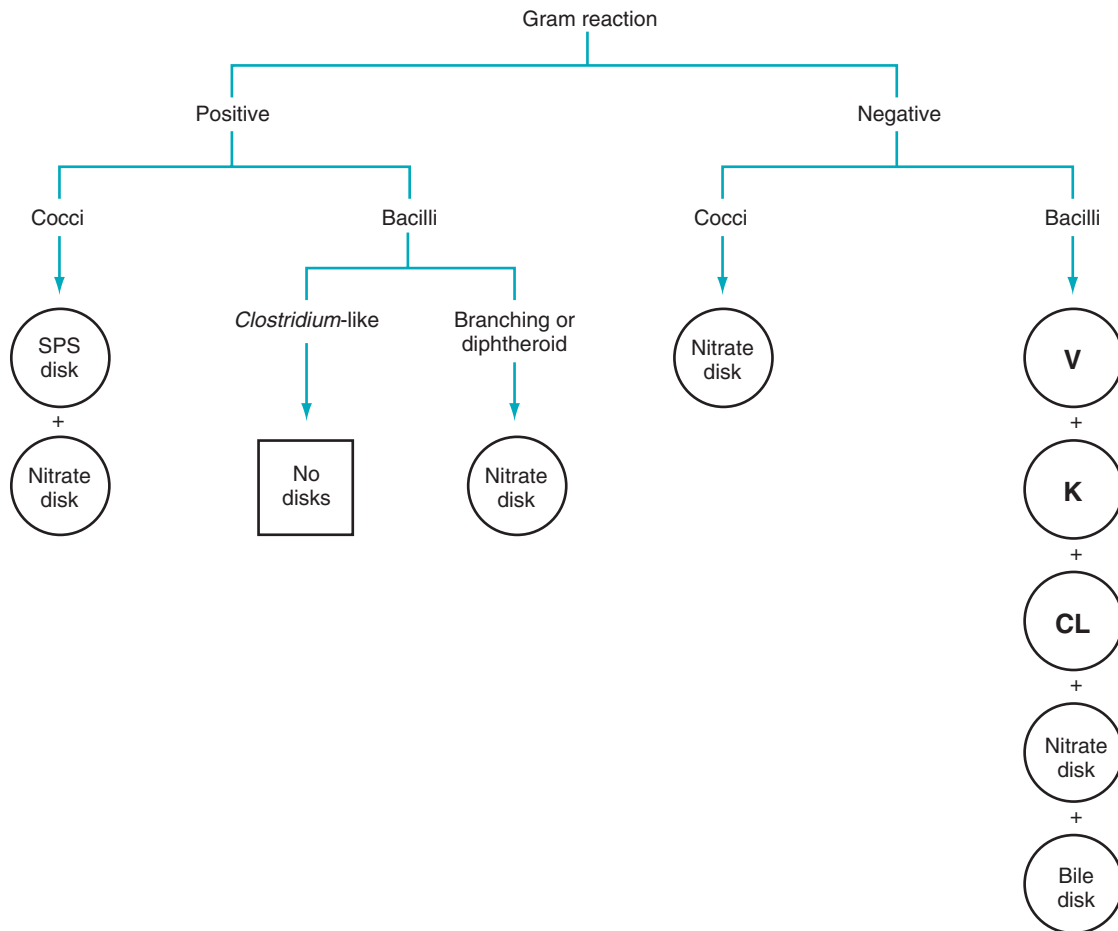


Fig. 22.14 Disks to add to the pure culture–subculture plate. *Clostridium*-like organisms are large, unbranched, gram-positive rods, with or without spores. Not all clostridia are large, and not all clostridia will stain as gram-positive rods. CL, Colistin (10 µg); K, kanamycin (1000 µg); SPS, sodium polyanethol sulfonate; V, vancomycin (5 µg).

The disks are placed on the heavily inoculated area of the plate, usually the first quadrant of the subculture plate. Disks of the proper potency must be pressed firmly to the surface of the plate to ensure uniform diffusion of the agent into the medium. The disk results for *C. ramosum* are shown in Fig. 22.15. Other disks, as described later, may be used for identification purposes. Disk results are interpreted as shown in Table 22.12.

Sodium polyanethol sulfonate disk

A sodium polyanethol sulfonate (SPS) disk aids in the identification of anaerobic gram-positive cocci. An SPS-sensitive, gram-positive anaerobic coccus can be presumptively identified as *Peptostreptococcus anaerobius*, whereas an SPS-resistant, spot indole-positive, gram-positive anaerobic coccus can be presumptively identified as *Peptoniphilus asaccharolyticus*.

Nitrate disk

The nitrate reduction disk test is a miniaturized version of the conventional nitrate reduction test. This determines an organism's ability to reduce nitrate to nitrite or nitrogen gas.

Bile disk

This disk can be used to determine an organism's ability to grow in the presence of relatively high concentrations (20%) of bile; however, it is not necessary if BBE agar is used as a



Fig. 22.15 Typical special potency antimicrobial disk results for *Clostridium ramosum*, susceptible to vancomycin (left) and kanamycin (right) and resistant to colistin (center). (Courtesy Anaerobe Systems, Morgan Hill, CA.)

primary plating medium. The bile disk may be added to the anaerobic subculture plate whenever the Gram stain reveals the isolate to be a gram-negative bacillus. Good growth on a BBE agar plate or growth in 20% bile indicates bile tolerance. A bile-tolerant, gram-negative anaerobic bacillus indicates that the isolate is likely a member of the *B. fragilis* group.

Table 22.12 Interpretation of special potency antimicrobial disk results

Vancomycin ^a	Kanamycin ^b	Colistin ^c	Interpretation
S	V	R	Probably a pink-staining, gram-positive bacillus such as <i>Clostridium ramosum</i> or <i>C. clostridioforme</i> ; however, if the kanamycin result is resistant, it could be a <i>Porphyromonas</i> sp.
S	R	R	<i>Porphyromonas</i> sp.
R	R	R	Probably a member of the <i>Bacteroides fragilis</i> group, <i>Parabacteroides</i> sp., or <i>Alistipes</i> sp. but could be a <i>Prevotella</i> sp..
R	V	V	<i>Prevotella</i> sp.
R	R	S	Probably a <i>Prevotella</i> sp.
R	S	S	<i>Bilophila wadsworthia</i> , <i>Fusobacterium</i> sp., <i>Sutterella</i> sp., or <i>Tannerella</i> sp.

^aVancomycin, 5μg.^bKanamycin, 1000μg.^cColistin, 10μg.

R, Resistant; S, susceptible (a zone of inhibition ≥10 mm is considered susceptible); V, variable.

From Conrads, G., et al. (2019). *Bacteroides*, *Porphyromonas*, *Prevotella*, *Fusobacterium*, and other anaerobic gram-negative rods. In K. C. Carroll, et al. (Eds). *Manual of clinical microbiology* (12th ed., p. 997). Washington, DC: ASM Press.

Fig. 22.16 Positive lecithinase reaction on egg yolk agar. The reaction occurs within the agar. *Clostridium perfringens* is shown here. (Courtesy Anaerobe Systems, Morgan Hill, CA.)

Lecithinase, lipase, and proteolytic reactions

An EYA plate can be used to determine the activities of lecithinase, lipase, and proteolytic enzymes. These reactions are of value in identifying many species of clostridia. Lecithinase cleaves lecithin found in egg yolk, releasing insoluble fat (diglyceride) that produces an opaque zone around the colony. This opacity is actually in the medium and is not a surface phenomenon (Fig. 22.16). Lipases hydrolyze triglycerides and diglycerides to fatty acids and glycerol. Lipase-positive organisms produce a colony covered with an iridescent, multicolored sheen, sometimes described as resembling the appearance of gasoline on water or mother of pearl. This multicolored sheen also can appear on the surface of the agar in a narrow zone around the colony. In contrast with the lecithinase reaction, the lipase reaction is essentially a surface phenomenon (Fig. 22.17).

Organisms that produce proteolytic enzymes (proteases) have a completely clear zone, often quite narrow, around their colonies. Proteolysis is best observed by holding the plate up to a strong light source. It is reminiscent of the complete clearing seen with β -hemolytic organisms on SBA plates.



Fig. 22.17 Positive lipase reaction on egg yolk agar. The reaction occurs on the surface of colonies and surrounding medium. A positive reaction by *Fusobacterium necrophorum* is shown here. (Courtesy Anaerobe Systems, Morgan Hill, CA.)

Presumptive identification of gram-positive anaerobes

Some gram-positive anaerobes can also be presumptively identified with simple procedures (Table 22.13). None of these organisms will grow on BBE agar or LKVB agar medium, so identification is based on the colony appearance on nonselective anaerobic blood agar and EYA.

- Large, irregular-shaped colonies on SBA demonstrating a double zone of β -hemolysis can be identified as *C. perfringens* (Fig. 22.18). These organisms will stain as large, boxcar-shaped bacilli.
- Large, flat colonies that produce a so-called *barnyard* or *horse stable* odor and fluoresce chartreuse (brilliant yellow-green) under long-wave UV light can be identified as *C. difficile*.

Table 22.13 Presumptive identification of gram-positive anaerobes*

Identification	Colony morphology on blood agar	Cellular morphology	Spot indole
<i>Clostridioides difficile</i>	Large, flat colonies; barnyard odor, characteristic fluorescence	Thin rods, rare spores	Negative
<i>Clostridium perfringens</i>	Large, irregular-shaped, double zone of β -hemolysis	Boxcar, large, square rods	Not done
<i>C. septicum</i>	Smoothly swarming	Thin rods, subterminal spores	Negative
<i>C. sordellii</i>	Very large, lobate, irregular, flat	Thin rods, subterminal spores	Positive
<i>C. tetani</i>	Smoothly swarming but slow growing	Swollen terminal spores	Positive
<i>Cutibacterium acnes</i>	Small, opaque, enamel white, circular (catalase-positive)	Coryneform rods	Positive
AGPC	Small, peaked, circular	Cocci, pairs and chains	Varies depending on species

*Additional information about presumptive identification of aerobes and facultative anaerobes can be found in Clinical and Laboratory Standards Institute. (2008). *Abbreviated identification of bacteria and yeast: approved guidelines (2nd ed.)*. CLSI document M35-A2. Wayne, PA: Clinical and Laboratory Standards Institute. AGPC, Anaerobic gram-positive cocci.

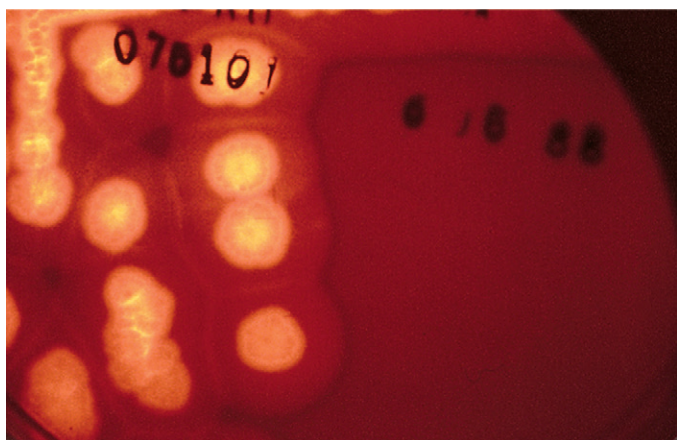


Fig. 22.18 Double zone of hemolysis produced by *Clostridium perfringens*—inner zone of complete β -hemolysis and outer zone of partial β -hemolysis. (Courtesy Anaerobe Systems, Morgan Hill, CA.)

- A rapidly growing colony exhibiting smooth swarming (as opposed to the waves observed with *Proteus*) and staining as thin rods with subterminal spores is likely to be *C. septicum*. Colonies often show β -hemolysis at 48 hours.
- Small, peaked, circular colonies appearing after 24 hours that stain as gram-positive cocci can be considered *Peptostreptococcus* spp. The CLSI M35-A2 document suggests continued use of the terms *Peptostreptococcus* spp. or *anaerobic gram-positive cocci* as a presumptive identification rather than identifying these organisms with the newer nomenclature.
- *Peptococcus niger* produces colonies that are initially black to olive green and become light gray when exposed to air, but it is only rarely isolated from clinical specimens.
- Small, opaque colonies that are catalase and indole positive and stain as coryneform rods can be identified as *C. acnes*.

Case check 22.3

In the Case in Point, a Gram-stained smear of the patient's leg wound revealed boxcar-shaped, gram-positive rods suggestive of clostridia. The presence of large gram-positive bacilli with colonies exhibiting a double zone of β -hemolysis in the absence of organisms recovered on aerobic cultures of the specimen is sufficient to report a presumptive identification of *C. perfringens*.

Presumptive identification of gram-negative anaerobes

Using Gram stain results, growth characteristics on primary plating media (e.g., nonselective anaerobic blood agar, BBE agar, KVLB agar), and a few rapid tests, many gram-negative anaerobes can be presumptively identified, often within 24 hours of inoculation (Table 22.14).

- Growth of large (>1 mm) gray-black colonies on BBE agar with growth on KVLB agar after an overnight incubation is sufficient to identify an isolate as a member of the *B. fragilis* group (Fig. 22.19).
- Translucent pitting colonies observed on the anaerobic blood agar plate, with no growth observed on BBE or KVLB agar, are characteristic of *Campylobacter (Bacteroides) ureolyticus*.
- Translucent colonies with a black fish-eye center observed on BBE agar (usually at 48 to 72 hours) can be used to presumptively identify *Bilophila wadsworthia* (Fig. 22.20).
- Ground-glass or breadcrumb-like colonies of long, slender, gram-negative rods with pointed ends are usually *Fusobacterium nucleatum* (Fig. 22.21).
- Organisms that grow on anaerobic blood agar and KVLB agar but not BBE agar and fluoresce brick red can be identified as *Prevotella*.
- Small, translucent or opaque colonies of tiny gram-negative cocci or diplococci can be identified as *Veillonella*.

Definitive identification of anaerobic isolates

For sterile site and some surgical isolates, it is important for the laboratory to provide full identification. A variety of

Table 22.14 Presumptive identification of gram-negative anaerobes^a

Identification	Colony morphology on blood agar or KVLB agar	Colony morphology on BBE agar	Cellular morphology	Spot indole
<i>Bacteroides fragilis</i> group	Large (>1 mm)	Large, convex, black gray	Regular	Not done
<i>Campylobacter ureolyticus</i>	Translucent, pitting (some)	No growth	Tiny rods or coccobacilli	Negative
<i>Bilophila wadsworthia</i>	Tiny, translucent	Translucent, with black center at 72 hours	Regular to filamentous	Negative
<i>Fusobacterium nucleatum</i>	Ground glass or breadcrumb	No growth	Fusiform, thin with pointed ends	Positive
<i>Porphyromonas</i>	Small, translucent or opaque, brick red fluorescence on blood agar, no growth on KVLB agar	No growth	Tiny coccobacilli	Positive
<i>Prevotella intermedia</i>	Small, translucent or opaque, brick red fluorescence on blood agar and KVLB agar	No growth	Tiny coccobacilli	Positive
<i>Veillonella</i>	Small, translucent or opaque, brick red fluorescence on blood agar, no growth on KVLB agar	No growth	Tiny diplococci	Negative

^aAdditional information about presumptive identification of aerobes and facultative anaerobes can be found in Clinical and Laboratory Standards Institute. (2008). *Abbreviated identification of bacteria and yeast: approved guidelines* (2nd ed.). CLSI document M35-A2. Wayne, PA: Clinical and Laboratory Standards Institute.

BBE, *Bacteroides bile esculin*; KVLB, kanamycin and vancomycin with laked sheep blood.



Fig. 22.19 Appearance of *Bacteroides fragilis* on a kanamycin–vancomycin–laked blood agar (left) and *Bacteroides bile esculin* (BBE) agar (right) biplate. Darkening of the BBE agar medium is the result of esculin hydrolysis. (Courtesy Anaerobe Systems, Morgan Hill, CA.)

techniques can be used to make a definitive identification (see Table 22.9). Many clinical microbiology laboratories use one of the commercially available biochemical-based or preexisting enzyme-based minisystems for making definitive identifications, but it is important to remember that none of these will identify all anaerobes that could potentially be isolated from clinical specimens. Most are designed to identify anaerobes that are most frequently encountered in clinical specimens. It is far more important for a system to identify these organisms than to identify obscure anaerobes that are only rarely isolated from clinical specimens or involved in infectious processes.

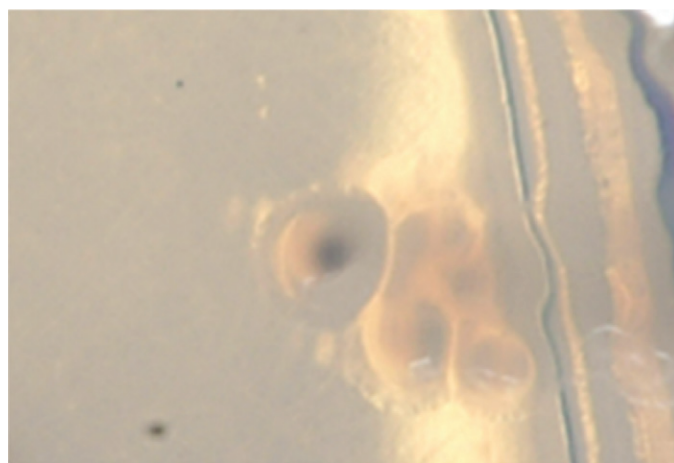


Fig. 22.20 Appearance of *Bilophila wadsworthia* on *Bacteroides bile esculin* agar. Note the fish-eye appearance of the colonies.

Biochemical-based multitest systems

A commercially available alternative to conventional tubed media is the biochemical-based identification system Analytical Profile Index (API; bioMérieux, Durham, NC). The API 20A system provides many of the same tests as conventional anaerobic systems but in the form of a plastic strip. Other systems are commercially available. The biochemical-based multitest systems are easier and faster to inoculate than a conventional system of plates and tubes. They require anaerobic incubation and larger model anaerobic jars. Some of the commercially available bags and pouches can be used to incubate biochemical-based trays and strips if an anaerobic chamber is not available. After 24 to 48 hours of incubation, test results are read, a code number is generated for each isolate, and identification is determined from an electronic database. It should be noted that the databases might not contain all the anaerobes that could be isolated from clinical specimens.

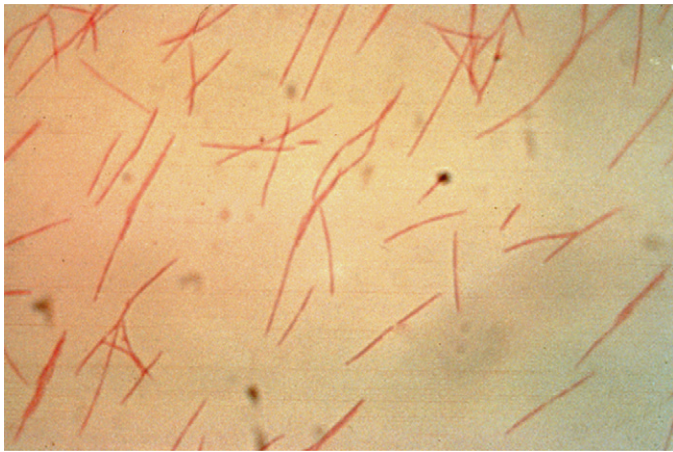


Fig. 22.21 Gram-stained appearance of *Fusobacterium nucleatum* subsp. *nucleatum* illustrating the fusiform morphology of this organism ($\times 1000$). (Courtesy S. L. Bartley, J. D. Howard, and R. Simon, Centers for Disease Control and Prevention, Atlanta, GA.)

Preformed enzyme-based systems

Many commercial identification systems are based on the presence of preformed (preexisting) bacterial enzymes. Because these systems do not depend on enzyme induction or bacterial growth, the results are available in about 4 hours. The small plastic panels or cards are easy to inoculate at the bench and do not require anaerobic incubation. Most of the systems generate code numbers, which are referenced in a manufacturer-supplied database. Like the biochemical-based multitest systems, these systems are primarily of value for identifying only the most commonly isolated anaerobes. One potential pitfall with these systems is that the database is sometimes divided by Gram-staining characteristics of the organism. Hence, it is vital to have an accurate Gram stain interpretation to arrive at an accurate identification. Special potency disks to determine the true Gram stain reaction and morphology of an organism can play a major role in the use of the preformed enzyme-based identification systems.

Preexisting enzyme-based systems include the VITEK ANI card (bioMérieux), AN-IDENT (bioMérieux), MicroScan rapid anaerobe identification panel (Beckman Coulter, Brea, CA), BBL Crystal anaerobe identification system (BD Diagnostic Systems), and the RapID-ANA II system (Thermo Fisher Scientific, Waltham, MA) shown in Fig. 22.22. In general, these systems use the same or similar substrates. They contain a number of nitrophenyl and naphthylamide compounds, colorless substances that produce yellow or red products, respectively, in the presence of appropriate enzymes. The results of some of these systems can be read via instrumentation. In performance evaluation studies, preformed enzyme-based and biochemical-based multitest systems were reported to have difficulty identifying gram-positive non-spore-forming bacilli, gram-positive anaerobic cocci, and some clostridia. For this reason, these methods are being replaced by MALDI-TOF mass spectrometry.

Cellular fatty acid analysis by high-resolution gas-liquid chromatography

Cellular fatty acid analysis is another method of identifying anaerobes. The term *cellular fatty acid* refers to fatty acids and

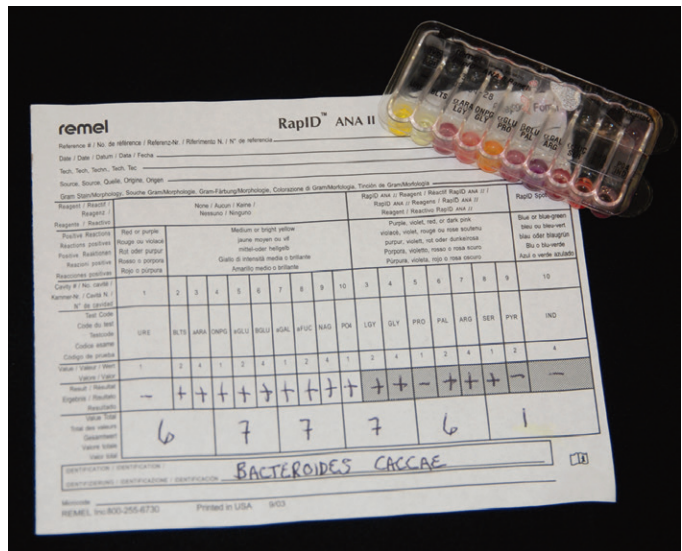


Fig. 22.22 RapID ANA-II preformed enzyme system (Thermo Fisher Scientific, Waltham, MA). This is one of many systems available for rapid definitive identification of commonly isolated anaerobes.

related compounds (e.g., aldehydes, hydrocarbons, dimethylacetals) present within organisms as cellular components. Cellular fatty acids are coded for on bacterial chromosomes, as opposed to plasmids, and are not affected by simple mutations or plasmid loss. Thus the fatty acid composition of a particular organism is relatively stable when the organism is grown under specific growth conditions—medium, incubation temperature, and time. Although fatty acid profiles can be identified manually, computerized, high-resolution gas-liquid chromatography (GLC) and specialized software programs are available to analyze cellular fatty acids of unknown bacteria and compare the results with patterns of known species.

The Sherlock microbial identification system (MIDI, Newark, DE) is a fully automated gas chromatography system that can be used to identify bacteria (including anaerobes) and yeasts (see Chapter 11). The organism is grown in pure culture on standardized medium. The bacterial cells are saponified to release the fatty acids from the bacterial lipids. After extraction, the methyl esters are analyzed by GLC. A chromatogram depicting the unknown organism's fatty acid composition is compared with a computer database of fatty acids of known anaerobes. Because no subjective interpretations are involved, the identifications are objective and highly reproducible.

Matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry

Most recently, bacterial identification has been revolutionized by the use of mass spectrometry. Specifically, a technique called *matrix-assisted laser desorption/ionization–time-of-flight* (MALDI-TOF) mass spectrometry measures the mass-to-charge ratio of ionized particles such as proteins from an organism (see Chapter 11). The technique produces a proteomic fingerprint that can be compared with reference strains. Results can be obtained in minutes, and this process has the potential of rapid identification of anaerobes without the need for an

aerotolerance test to be performed. However, the database for anaerobes is still somewhat limited, with only the most commonly encountered anaerobes identified.

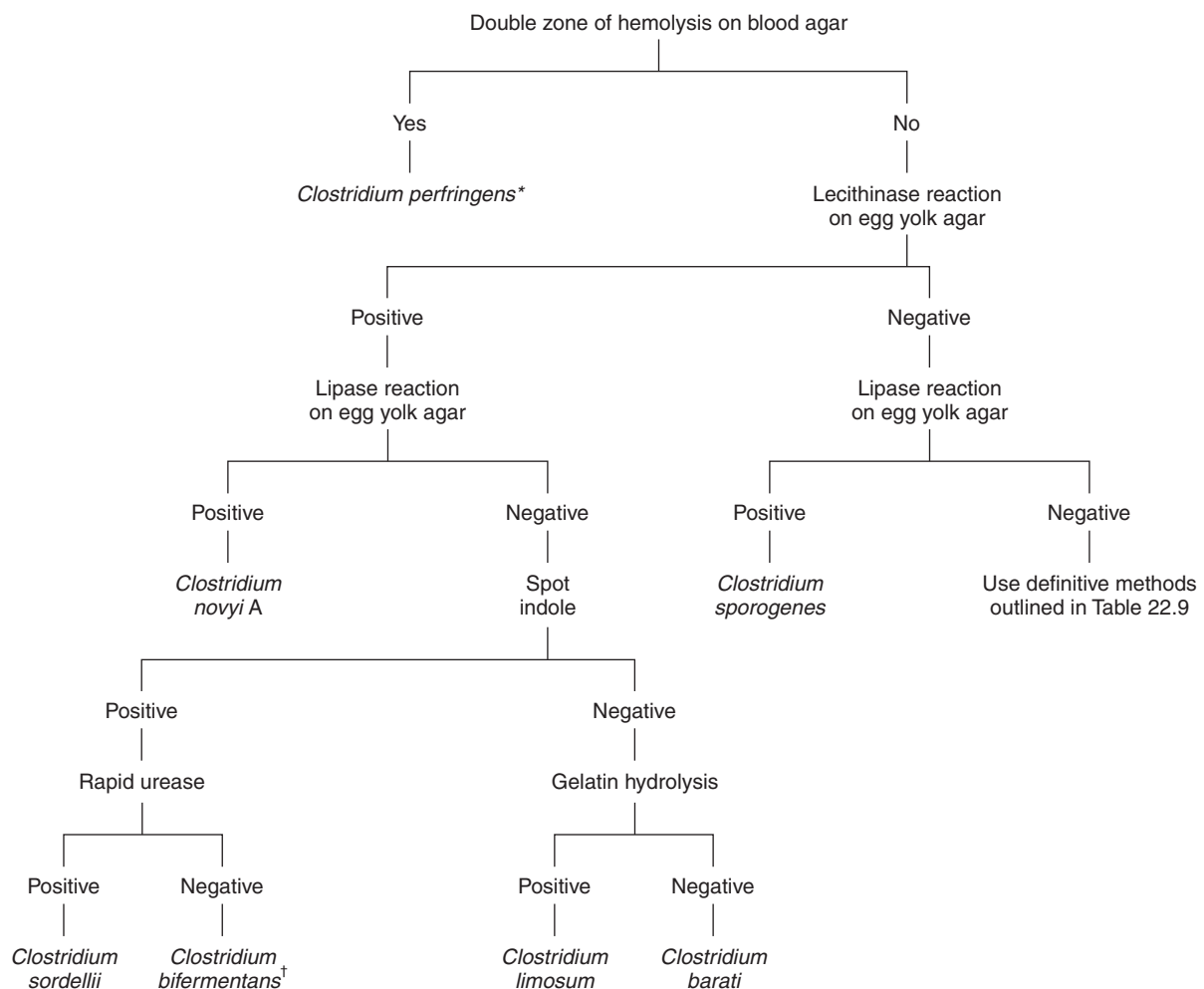
16S Ribosomal RNA gene sequencing

Anaerobes that are difficult to identify often can be definitively identified using 16S rRNA gene sequencing. The 16S rRNA gene, ribosomal deoxyribonucleic acid (rDNA), has highly conserved and highly variable regions that can be used for microbial identification. The DNA is first extracted from the organism; the target segment of about 500 base pairs is amplified through polymerase chain reaction technology and then sequenced on an automated sequencer. The resulting nucleotide sequence is compared with known sequences in public databases such as GenBank, a repository of sequences maintained by the National Institutes of Health. The MicroSeq microbial identification system (Applied Biosystems, Foster City, CA) has kits and an electronic database that can be used for bacterial or fungal identification. The results can be obtained in approximately 5 hours.

Identification of *Clostridium* and *Clostridioides* species

An identification algorithm for some *Clostridium* spp. is shown in Fig. 22.23. Identification of clinically encountered clostridia using cultural and biochemical characteristics is summarized in Table 22.15. The clostridial cell wall structure is similar to that of other gram-positive bacteria. However, some species appear gram variable, and some (e.g., *C. ramosum* and *C. clostridioforme*) routinely stain gram negative. The vancomycin special potency antimicrobial disk set up at the same time as the aerotolerance test is used to determine the true Gram stain reaction of a pink-staining anaerobic bacillus. Gram-positive clostridia are always susceptible to vancomycin.

Certain cultural characteristics can be used initially to identify clostridial isolates. As noted, a boxcar-shaped, gram-positive anaerobic bacillus that produces a characteristic double zone of hemolysis on SBA can be presumptively identified as *C. perfringens*. A double zone of hemolysis appears as an inner zone of complete β -hemolysis and an outer zone of



*Boxcar-shaped bacilli; lecithinase positive; reverse Christie, Atkins, Munch-Petersen (CAMP) test positive; subterminal spores, but spores rarely observed.

†Produces chalk-white colonies on egg yolk agar.

Fig. 22.23 Identification of *Clostridium* spp. Use this chart for organisms fulfilling the following three criteria: (1) anaerobic, (2) gram positive, and (3) spore former. (From Mangels, J. I. [1998]. *Anaerobic bacteriology*. In H. D. Isenberg [Ed.], *Essential procedures for clinical microbiology*. Washington, DC: American Society for Microbiology.)

Table 22.15 Characteristics of some clinically encountered *clostridia*^a

<i>Clostridium</i> species	Aerotolerant growth	Swarming	Double-zone β -hemolysis	Chartreuse fluorescence	Gram-stain reaction	Spore position	Motility	Indole	Lecithinase	Lipase	Proteolysis in milk	Gelatin hydrolysis	Acid from lactose	Urease
<i>C. bifermentans</i>	–	–	–	–	+	ST	+	+	+	–	+	–	–	–
<i>C. clostridioforme</i>	–	–	–	–	–	ST	– ⁺	–	–	–	–	–	+	–
<i>C. difficile</i> ^a	–	–	–	+	+	ST	+	–	–	–	–	– ⁺	–	–
<i>C. novyi</i> type A	–	–	–	–	+	ST	– ⁺	–	+	+	–	+	–	–
<i>C. perfringens</i>	–	–	+	–	+	– ST	–	–	+	–	–	+	+	–
<i>C. ramosum</i>	–	–	–	–	–	(T)	–	–	–	–	–	–	+	–
<i>C. septicum</i>	–	+	–	–	+	ST	+	–	–	–	–	–	+	–
<i>C. sordellii</i>	–	–	–	–	+	ST	+	+	+	–	+	–	–	+
<i>C. sporogenes</i>	–	–	–	–	+	ST	+	–	– ⁺	+	+	– ⁺	–	–
<i>C. tertium</i>	+	–	–	–	+	T	+	–	–	–	–	–	+	–
<i>C. tetani</i>	–	+	–	–	+	T	+	– ⁺	–	–	–	+	–	–

^aCurrently named *Clostridioides difficile*.ST, Subterminal; T, terminal; (T), variable but usually terminal; –ST, usually not observed but subterminal when seen; +[–], most strains positive; –⁺, most strains negative.Modified from Engelkirk, P. G., et al. (1992). *Principles and practice of clinical anaerobic bacteriology*. Belmont, CA: Star Publishing.

partial β -hemolysis because of two different hemolysins (see Fig. 22.18). A swarming, gram-positive, anaerobic bacillus with terminal spores is probably *C. tetani*, whereas one with subterminal spores is most likely *C. septicum*. EYA is useful for detecting lecithinase, lipase, and proteolytic enzymes produced by some clostridia. Lecithinase-positive clostridia include *C. bifermentans*, *C. sordellii*, *C. perfringens*, and *C. novyi* type A. Some lipase-positive clostridia are *C. botulinum*, *C. novyi* type A, and *C. sporogenes*.

C. sordellii is the only member of the clostridia to exhibit urease activity. *C. bifermentans* and *C. sordellii* are spot indole positive. Finally, it is important to remember that *C. tertium* will grow minimally on the aerotolerance plate and can be mistaken for a facultative anaerobe. However, growth on the anaerobic subculture plate will exhibit much heavier growth than on the aerotolerance test plate.

Clostridioides difficile can be recovered from feces by inoculating a cycloserine–cefotaxime–fructose agar (CCFA) plate. CCFA is a selective and differential medium for the recovery and presumptive identification of *C. difficile*. On CCFA, *C. difficile* produces yellow ground-glass colonies; the originally pink agar turns yellow in the vicinity of the colonies because of the fermentation of fructose. In reduced conditions, the indicator, neutral red, turns yellow in the presence of an acid pH. Although other organisms may grow on CCFA, their colonies are smaller and do not resemble the characteristic colonies of *C. difficile*. In addition, *C. difficile* has a characteristic odor, resembling a horse stable, and colonies on blood agar fluoresce chartreuse under UV light.

Organisms that are isolated must be tested for toxin production because a small percentage of the human population can carry *C. difficile* as normal biota, and some isolates may not be toxin producers. Culture for *C. difficile* has largely been replaced by assays designed to detect the toxins produced by the organism in feces or nucleic acid amplification tests (NAATs). The cell culture cytotoxicity assay detects toxin B in fecal samples by tissue culture; however, this assay is technically demanding and can take 2 to 3 days for results to be obtained. A number of rapid detection tests are available that can detect toxin A, toxin B, the enzyme glutamate dehydrogenase (GDH), or a combination of *C. difficile* toxins and GDH. Many of the kits use enzyme immunoassay (EIA) methods. Glutamate dehydrogenase is not a virulence factor, but it is an enzyme frequently associated with *C. difficile*. A negative glutamate dehydrogenase assay can be used as rapid screening to rule out CDAD; however, a positive glutamate dehydrogenase assay should be confirmed as positive for *C. difficile* toxins by more specific assays. NAATs, either as a stand-alone test or as part of a multiplex diarrheal pathogen panel, can rapidly determine the presence of toxin A and toxin B genes in feces and are rapidly becoming the new gold standard for the detection of toxin-producing *C. difficile*.

A study of 81 patients with symptoms suggestive of CDAD compared three NAATs and two non-NAATs for the detection of CDAD. The authors reported sensitivities of 88.5% to 96.2% for the NAATs and 42.3% and 61.5% for the non-NAATs. The NAATs detected 34.7% more cases

Table 22.16 Characteristics of some clinically encountered gram-positive non-spore-forming anaerobic bacilli

	48-Hour Colony (<1 mm)	Red pigment colony	β -Hemolytic colony	Rough colony	Branched bacilli	Catalase	Indole	Comments
<i>Actinomyces israelii</i>	+	–	–	+	+	–	–	Molar tooth colony, slow growth
<i>A. meyeri</i>	– ⁺	–	–	–	+	–	–	
<i>A. naeslundii</i>	– ⁺	–	–	– ⁺	+	–	–	
<i>A. odontolyticus</i>	–	+	–	–	+	–	–	
<i>A. viscosus</i>	–	–	–	– ⁺	+	+	–	
<i>Bifidobacterium</i> spp.	–	–	–	–	–	–	–	Rods with forked ends
<i>Eggerthella lenta</i>	–	–	–	–	–	V	–	Nitrate reduction positive
<i>Eubacterium</i> spp.	–	–	–	–	–	–	–	Nitrate reduction negative
<i>Cutibacterium acnes</i>	–	–	–	–	–	+	+	Nitrate reduction positive
<i>Pseudopropionibacterium propionicum</i>	+	–	–	+	+	–	–	Nitrate reduction positive

V, Variable; –⁺, most strains negative.

Modified from Engelkirk, P. G., et al. (1992). *Principles and practice of clinical anaerobic bacteriology*. Belmont, CA: Star Publishing.

of CDAD than the non-NAAT methods. The specificity for all five assays ranged from 96.4% to 100%. Because patients with formed stools are rarely positive for CDAD, EIA and NAATs should not be performed on these types of specimens.

Identification of anaerobic non-spore-forming, gram-positive bacilli

Identifying characteristics of clinically encountered non-spore-forming, gram-positive bacilli are listed in Table 22.16. In general, non-spore-forming, gram-positive bacilli are difficult to identify because the preformed enzyme system databases often do not contain many of the less commonly isolated species. Sequencing of the 16S rDNA gene has been used recently to reclassify many of these organisms into new genera. MALDI-TOF mass spectrometry databases are being continually improved to identify these organisms.

Actinomyces spp.

Actinomyces spp. are straight to slightly curved bacilli of differing lengths, from short rods to long filaments. Short rods may have clubbed ends and may be seen in diphtheroid arrangements, short chains, or small clusters. Longer rods and filaments may be straight or wavy and branched. Although the *Actinomyces* are gram positive, irregular staining can produce a beaded or banded appearance, much like that seen with *Nocardia* spp. The typical branching, filamentous, Gram-stained appearance of an *Actinomyces* sp., depicted in Fig. 22.2, is referred to as *Actinomyces*-like.

Members of the genus *Actinomyces* are seldom obligate anaerobes. However, some are fastidious, requiring special vitamins, amino acids, and hemin for adequate growth.

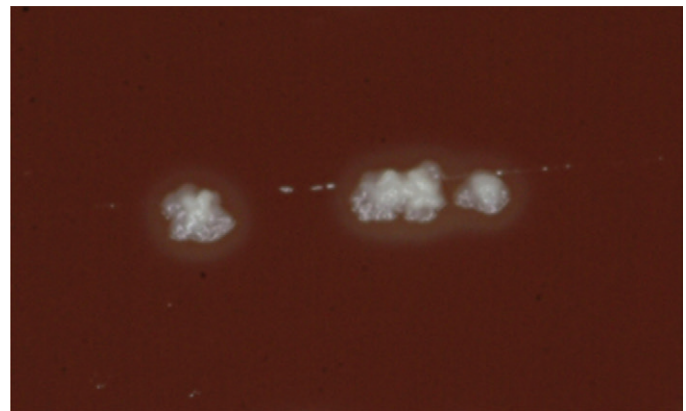


Fig. 22.24 Appearance of *Actinomyces israelii* showing the molar tooth colonies typical for this anaerobe.

Young *Actinomyces* colonies are frequently spiderlike or wooly, whereas older colonies of *A. israelii* usually have a molar tooth appearance (Fig. 22.24). Depending on the species, colonies may be red, pink, tan, yellow, white, or grayish. Approximately 25 species have been isolated from human specimens.

Bifidobacterium spp.

Bifidobacterium spp. are variable in shape, ranging from coccobacilli to long branching rods. The ends of the cells may be pointed, bent, club-shaped, spatulated, or bifurcated (forked). Cells may appear singly or in chains and as starlike aggregates, V arrangements, or palisade clusters. Colonies of *Bifidobacterium* spp. are convex, entire, and cream to white, smooth, glistening, and soft.

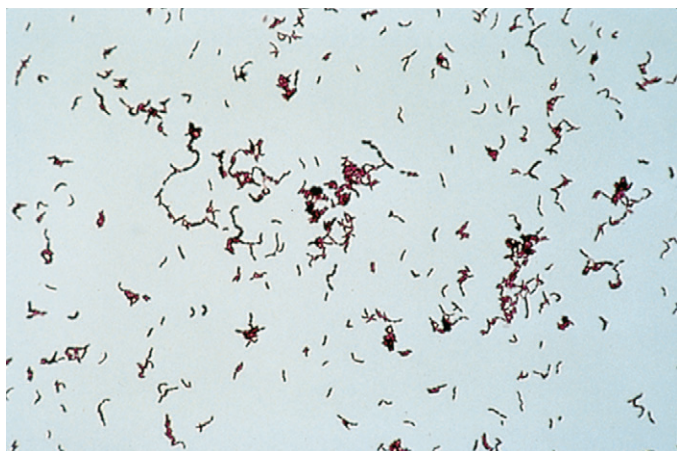


Fig. 22.25 Gram-stained appearance of *Cutibacterium acnes*, illustrating the term diphtheroid ($\times 1000$). (Courtesy S. L. Bartley, J. D. Howard, and R. Simon, Centers for Disease Control and Prevention, Atlanta, GA.)

Lactobacillus spp.

Colony morphology of the lactobacilli is greatly varied, with some species appearing as pinpoint α -hemolytic colonies on SBA. Others have been described as medium in size, with a rough appearance and gray color. Lactobacilli are catalase negative and, unless a Gram stain is performed, differentiation from *Streptococcus* sp. viridans group is difficult.

Propionibacterium and Cutibacterium spp.

Propionibacterium and *Cutibacterium* spp. are pleomorphic rods with a diphtheroid appearance. The Gram-stained appearance of *C. acnes* is shown in Fig. 22.25. Because *C. acnes* is a common member of the skin microbiota, it is frequently isolated from blood culture bottles as a contaminant. However, *C. acnes*, like coagulase-negative staphylococci, can cause subacute bacterial endocarditis, prosthetic joint infection, and bacteremia and thus is not always a contaminant. A gram-positive anaerobic diphtheroid that is both catalase and spot indole positive can be presumptively identified as *C. acnes*.

Pseudopropionibacterium propionicum can cause actinomycosis. This organism ranges considerably in size and shape, ranging from coccoid and short diphtheroidal rods to long, branched filaments. Individual cells may be of uneven diameter and have distended or clubbed ends. As the genus name implies, propionic acid is a major metabolic end product of *Pseudopropionibacterium* and *Propionibacterium* spp.

Identification of anaerobic gram-negative bacilli

Key tests for the identification of gram-negative anaerobic bacilli are listed in Table 22.17. Definitive identification of many of the commonly occurring gram-negative anaerobes can be accomplished by the preformed enzyme systems or MALDI-TOF mass spectrometry, so reliance on more extensive methods is usually not necessary.

Bacteroides spp.

The genus *Bacteroides* was divided previously into bile-tolerant and bile-sensitive species. However, most of the

bile-sensitive species were transferred to the genera *Prevotella* and *Porphyromonas*. Bile-tolerant species grow in the presence of 20% bile, so colonies will be present on BBE agar. They will exhibit robust growth on KVLB agar.

Bacteroides fragilis group and other bile-resistant Bacteroides

Bile-tolerant *Bacteroides* spp. include members of the *B. fragilis* group and *Parabacteroides* spp. Members of the *B. fragilis* group, which contains more than 50 species, are especially pathogenic. The most clinically relevant are *B. fragilis*, *B. thetaiotaomicron*, and *B. ovatus*. *B. fragilis* is the most common species of anaerobic bacteria isolated from infectious processes of soft tissue and anaerobic bacteremia and is responsible for more than 60% of infections caused by the *B. fragilis* group. *B. thetaiotaomicron* is the next most frequently encountered member of the *B. fragilis* group and often exhibits the highest degree of antimicrobial resistance. Gram-stained smears of *Bacteroides* spp. colonies reveal gram-negative coccobacilli or bacilli, but cells in broth cultures are frequently pleomorphic. The Gram-stained appearance of a typical *Bacteroides* species is shown in Fig. 22.26.

Colonies of the *B. fragilis* group on a BBE agar plate are usually brown to black, on the originally pale yellow medium, and a minimum of 1 mm in diameter at 24 hours. Good growth is the result of bile tolerance, and darkening of the medium is caused by esculin hydrolysis. A dark precipitate (stippling) in the medium around the areas of heavy growth is suggestive of the species *B. fragilis*, although some strains of *Bacteroides ovatus* also cause stippling. The appearance of *B. fragilis* group organisms on a biplate of BBE agar and KVLB agar is shown in Fig. 22.19. Caution must be taken, however, when interpreting results obtained on BBE agar. *B. vulgatus*, a member of the *B. fragilis* group, often does not hydrolyze esculin and therefore may not produce a brown to black discoloration of the medium. Depending on the commercial source, age, and storage conditions of the medium, other organisms, such as *F. mortiferum*, *Klebsiella pneumoniae*, *Enterococcus* spp., and some yeasts, may also grow on BBE agar. Their colony size (tends to be smaller), Gram stain morphology, and aerotolerance will aid in the recognition of these other organisms.

Characteristics of the most commonly isolated members of the *B. fragilis* group can be found in Table 22.17. All members will be resistant to colistin, kanamycin, and vancomycin special potency disks. The members of the *B. fragilis* group can be divided into those that produce indole (e.g., *B. thetaiotaomicron*, *B. uniformis*, and *B. ovatus*) and those that do not (e.g., *B. fragilis*, *B. vulgatus*, and *B. caccae*). Catalase activity can be found in *B. fragilis*, *B. ovatus*, *B. thetaiotaomicron*, and *P. distasonis*.

Bilophila sp.

Bilophila wadsworthia is a bile-resistant anaerobe that will grow on BBE agar with a characteristic fish-eye appearance (see Fig. 22.20), and it also will grow on KVLB agar. The organism is strongly catalase positive and nitrate positive.

Prevotella spp.

Prevotella spp. will grow on KVLB agar but not on BBE agar. They are resistant to vancomycin and kanamycin but are variable in their susceptibility to the colistin special potency disk. Some species of *Prevotella* produce protoporphyrin, a dark pigment that causes their colonies to become brown to black with

Table 22.17 Phenotypic characteristics of anaerobic gram-negative bacilli

	Vancomycin	Kanamycin	Colistin	Bile resistant	Growth on KVLB agar	Esculin hydrolysis	Red fluorescence	Chartreuse fluorescence	Indole	Oxidase	Catalase	Urease	Lipase	Nitrate	Pitting colony	Formate-Fumarate	Comment
Bacteroides fragilis group																	
<i>B. fragilis</i>	R	R	R	+	+	+	-	-	-	-	+	-	-	-	-	-	
<i>B. caccae</i>	R	R	R	+	+	+	-	-	-	-	- ⁺	-	-	-	-	-	
<i>B. ovatus</i>	R	R	R	+	+	+	-	-	+	-	+	-	-	-	-	-	
<i>B. thetaiotaomicron</i>	R	R	R	+	+	+	-	-	+	-	+	-	-	-	-	-	
<i>B. uniformis</i>	R	R	R	+	+	-	-	-	+	-	V	-	-	-	-	-	
<i>B. vulgatus</i>	R	R	R	+	+	+	-	-	-	-	- ⁺	-	-	-	-	-	
Campylobacter ureolyticus group																	
<i>C. ureolyticus</i>	R	S	S	-	-	-	-	-	-	+	V	+	-	+	V	+	Pitted and nonpitted
<i>Campylobacter</i> spp.	R	S	S	V	-	-	-	-	-	-	-	-	-	+	V	+	Pitted and nonpitted
<i>Sutterella wadsworthensis</i>	R	S	S	+	-	-	-	-	-	-	-	-	-	+	V	+	Pitted and nonpitted
<i>Bilophila wadsworthia</i>	R	S	S	+	+	+	-	-	-	-	+	V	-	+	-	-	Fish eye on BBE
Parabacteroides																	
<i>P. distasonis</i>	R	R	R	+	+	+	-	-	-	-	+	-	-	-	-	-	
Prevotella																	
<i>P. bivia</i>	R	R	R	-	+	-	+	-	-	-	-	-	-	-	-	-	Delayed pigment
<i>P. disiens</i>	R	R	V	-	+	-	+	-	-	-	-	-	-	-	-	-	Delayed pigment
<i>P. denticola</i>	R	R	V	-	+	-	+	-	-	-	-	-	-	-	-	-	Brown colonies
<i>P. intermedia</i>	R	R	V	-	+	-	+	-	+	-	-	-	+	-	-	-	
<i>P. loescheii</i>	R	R	V	-	+	-	+	-	-	-	-	-	+	-	-	-	
<i>P. melaninogenica</i>	R	R	V	-	+	-	+	-	-	-	-	-	-	-	-	-	Brown colonies
Porphyromonas																	
<i>P. asaccharolyticus</i>	S	R	R	-	-	-	+	-	+	-	-	-	-	-	-	-	
<i>P. endodontalis</i>	S	R	R	-	-	-	+	-	+	-	-	-	-	-	-	-	
<i>P. gingivalis</i>	S	R	R	-	-	-	-	-	+	-	-	-	-	-	-	-	
Fusobacterium																	
<i>F. nucleatum</i>	R	S	S	-	-	-	-	+	+	-	-	-	-	-	-	-	Slender, pointed cells
<i>F. mortiferum</i>	R	S	S	+	+	-	-	+	-	-	-	-	-	-	-	-	Bizarre, round bodies
<i>F. necrophorum</i>	R	S	S	- ⁺	+	-	-	+	+	-	-	-	+-	-	-	-	Large, pleomorphic cells
<i>F. varium</i>	R	S	S	+	-	-	-	+	+	-	-	-	- ⁺	-	-	-	Large, rounded ends

BBE, *Bacteroides bile esculini*; KVLB, kanamycin and vancomycin with laked sheep blood; R, resistant; S, sensitive; V, strains are variable; +, most strains are positive, -⁺, most strains are negative.

Modified from Engelkirk, P. G., et al. (1992). *Principles and practice of clinical anaerobic bacteriology*. Belmont, CA: Star Publishing.

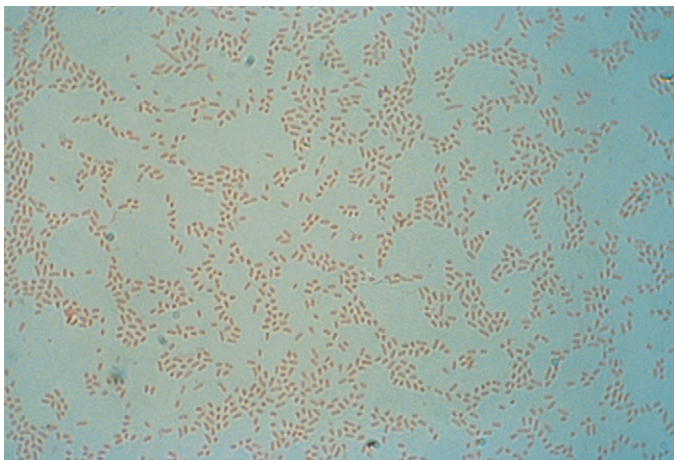


Fig. 22.26 Gram-stained appearance of *Bacteroides thetaiotaomicron*, illustrating the typical appearance of *Bacteroides* spp. (×1000). (Courtesy S. L. Bartley, J. D. Howard, and R. Simon, Centers for Disease Control and Prevention, Atlanta, GA.)

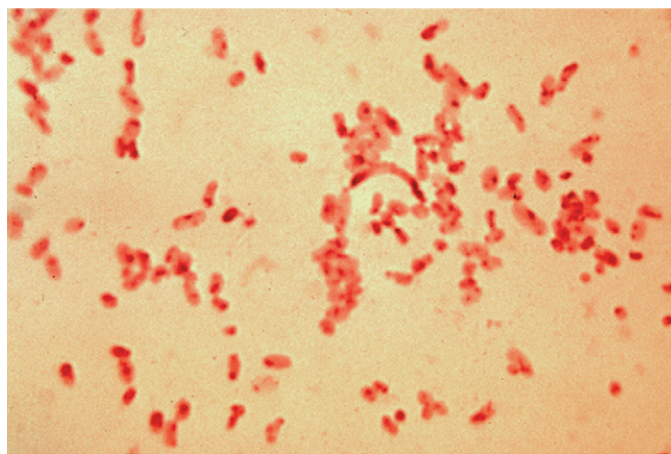


Fig. 22.27 Gram-stained appearance of *Fusobacterium mortiferum*, illustrating pleomorphism (×1000). (Courtesy S. L. Bartley, J. D. Howard, and R. Simon, Centers for Disease Control and Prevention, Atlanta, GA.)

age. Colony pigmentation may take 2 to 3 weeks of incubation before it becomes evident on routine Brucella blood agar plates, but it appears sooner on KVLB agar. Therefore nonpigmented colonies on KVLB agar or Brucella blood agar should be subjected to long-wave UV light, such as with a Wood lamp, to detect the typical brick red fluorescence of pigment-producing *Prevotella* spp. that appears before the brown pigment. The brick red fluorescence under UV light is similar to that shown in Fig. 22.13A. Some species of pigmented *Prevotella* fluoresce colors other than brick red (e.g., brilliant red, yellow, orange, pink orange), and some do not fluoresce at all. Only brick red fluorescence allows presumptive identification of the pigmented *Prevotella* group. *P. intermedia* and *P. loescheii* produce lipase, and *P. intermedia* is spot indole positive. In a Gram-stained smear, *Prevotella* spp. appear as gram-negative coccobacilli or bacilli, very similar to *Bacteroides* spp.

Porphyromonas spp.

Porphyromonas spp. will produce brick red fluorescence under UV light, similarly to *Prevotella*, but some species do not fluoresce. Because most *Porphyromonas* strains are susceptible to vancomycin, they will not grow on KVLB agar; however, in contrast with *Prevotella*, they are resistant to colistin with the special potency disks. Most *Porphyromonas* spp. are spot indole positive.

Campylobacter ureolyticus group

The *Campylobacter ureolyticus* group consists of bile-sensitive and bile-tolerant nonpigmented organisms (see Table 22.17). Reports have shown that the so-called *pitting anaerobes* of the *C. ureolyticus* group, which includes *C. ureolyticus*, *C. gracilis*, *C. curvus*, *C. rectus*, and *Sutterella wadsworthensis*, are actually microaerophiles rather than obligate anaerobes. One useful identifying characteristic of this group is the colony appearance. Many of the organisms have colonies that appear to pit the agar. However, not all strains pit agar, and among those strains that do, not all colonies will appear to be pitting, so they may resemble a mixed culture. Growth in broth is enhanced by the addition of formate or fumarate, a characteristic unique to this group. The members of this group are positive in the nitrate test, whereas *C. ureolyticus* is able to hydrolyze urea and is spot oxidase positive. *C. ureolyticus*

is bile sensitive, *Sutterella* spp. are bile tolerant, and the other campylobacters are variable in bile sensitivity.

Fusobacterium

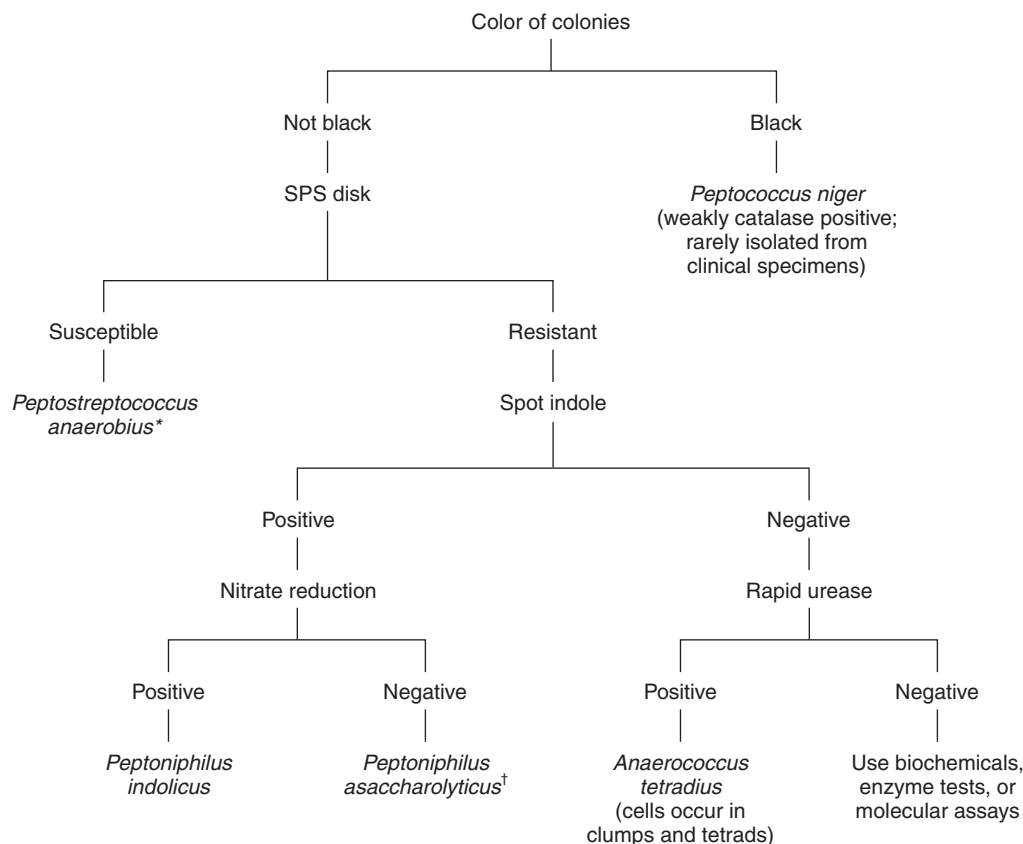
Fusobacterium spp. are often described microscopically as long, thin, tapered rods, a morphology characteristically referred to as *fusiform*. It is important to note, however, that only *F. nucleatum* has cells that are consistently fusiform in shape, and clinically encountered bacteria that are fusiform in shape are not necessarily *Fusobacterium*. The Gram-stained appearance of *F. nucleatum* is shown in Fig. 22.21. Other fusobacteria, such as *F. mortiferum*, appear pleomorphic and exhibit globular forms, swellings, and other bizarre shapes. The pleomorphism of *F. mortiferum* is depicted in Fig. 22.27. Organisms other than fusobacteria can also have fusiform-shaped cells, such as *C. gracilis*, *Bacteroides forsythus*, and microaerophilic *Capnocytophaga*.

Fusobacteria are resistant to vancomycin but susceptible to colistin and kanamycin with the special potency disks. However, all except *F. nucleatum* will grow on KVLB agar because the level of kanamycin is reduced from that of the special potency disk. With the exception of *F. mortiferum*, the fusobacteria are indole positive, and all will fluoresce chartreuse under long-wave UV light. In addition, *F. necrophorum* is positive for lipase when grown on EYA.

Identification of anaerobic cocci

An algorithm depicting the presumptive identification of the anaerobic gram-positive cocci is shown in Fig. 22.28; the Gram stain appearance of a typical anaerobic coccus is seen in Fig. 22.29. Phenotypic characteristics used to identify anaerobic cocci are listed in Table 22.18. As noted, presumptive identification of gram-positive anaerobic cocci can be reported as *Peptostreptococcus* spp. or as anaerobic gram-positive cocci. Full identification can be accomplished by preformed enzyme systems; 16S rRNA sequencing and MALDI-TOF mass spectrometry have been shown to identify accurately many of the more commonly isolated anaerobic gram-positive cocci.

A black-pigmented, anaerobic, gram-positive coccus can be identified as *Peptococcus niger*. *Peptostreptococcus anaerobius* can be identified by a zone of inhibition around an SPS disk.



*Some strains of *Peptostreptococcus micros* are susceptible to SPS, but they generally produce smaller zones of inhibition.

†*Anaerococcus* (*Peptostreptococcus*) *hydrogenalis* is also indole-positive but unlike *P. asaccharolyticus* is alkaline phosphatase positive.

Fig. 22.28 Identification of anaerobic gram-positive cocci. Anaerobic gram-positive cocci (AGPC) are susceptible to metronidazole, whereas microaerophilic gram-positive cocci are not. An 80- μ g metronidazole elution disk (BD Diagnostic Systems, Sparks, MD) can be used to determine metronidazole susceptibility. Although metronidazole-resistant strains of AGPC have been reported, they appear to be rare. SPS, Sodium polyanethol sulfonate.

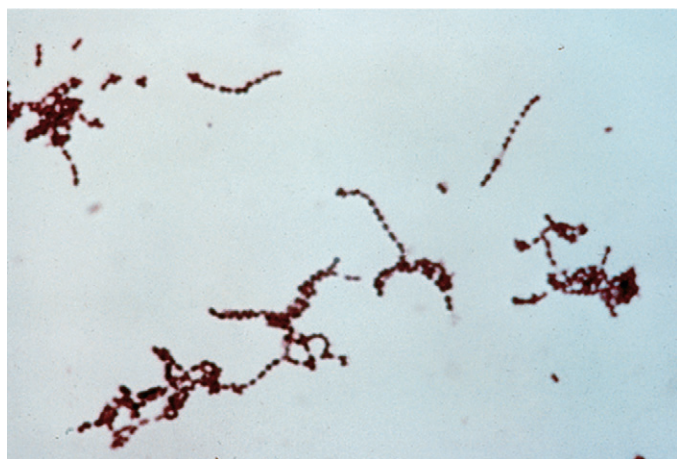


Fig. 22.29 Gram-stained appearance of a *Peptostreptococcus* sp. illustrating the chain formation that occurs with some species of anaerobic gram-positive cocci ($\times 1000$). (Courtesy S. L. Bartley, J. D. Howard, and R. Simon, Centers for Disease Control and Prevention, Atlanta, GA.)

Spot indole-positive isolates are often members of the genus *Peptoniphilus*, with *P. indolicus* identified by a positive nitrate test result. The only urease-positive anaerobic coccus is *Anaerococcus tetradius*. *Finegoldia magna* and *Parvoimonas micra* are similar in biochemical reactions but can be differentiated on the basis of

size; *F. magna* has cocci larger than $0.6\mu\text{m}$, whereas *P. micra* cocci are smaller. *Veillonella parvula* is the only commonly encountered gram-negative coccus, and it can be identified with Gram stain, a positive catalase reaction, and red fluorescence under long-wave UV light. It is also positive for nitrate reduction and is resistant to the vancomycin special potency disk.

Antimicrobial susceptibility testing

Traditionally, the isolation, identification, and susceptibility testing of anaerobes have been slow compared with those of aerobic bacteria isolated from the same specimen. As a result, when health care providers suspect an anaerobic infection, they routinely select a broad-spectrum agent for empiric therapy that will cover most anaerobes, pending outcome of the culture. This practice was based on the fact that many antimicrobial agents could be relied on to have good activity against the most commonly isolated anaerobes. However, resistance to antimicrobial agents has increased in recent years, and the susceptibility patterns of many anaerobes to certain antimicrobial agents can no longer be guaranteed. Antimicrobial resistance has mainly been observed in the *B. fragilis* group but has also been noted to a lesser extent in most of the clinically significant anaerobes.

Table 22.18 Characteristics of some clinically encountered anaerobic cocci

	Gram-stain reaction	Catalase	Indole	Urease	Nitrate reduction	Red fluorescence	Comments
<i>Peptostreptococcus anaerobius</i>	+	—	—	—	— ⁺	—	Inhibited by SPS disk
<i>Parvimonas micra</i>	+	—	—	—	—	—	
<i>Peptoniphilus asaccharolyticus</i>	+	— ⁺	+ ⁻	—	—	—	
<i>Peptoniphilus indolicus</i>	+	—	+	—	+	—	
<i>Finegoldia magna</i>	+	— ⁺	—	—	—	—	
<i>Anaerococcus prevotii</i>	+	— ⁺	—	—	—	—	
<i>Anaerococcus tetradius</i>	+	—	—	+	—	—	
<i>Veillonella parvula</i>	—	+	—	—	— ⁺	+ ⁻	

SPS, Sodium polyanethol sulfate; +⁻, most strains are positive; —⁺, most strains are negative.
 Modified from Engelkirk, P. G., et al. (1992). *Principles and practice of clinical anaerobic bacteriology*. Belmont, CA: Star Publishing.

CLSI document M11-A8, *Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria* (approved standard), provides guidelines for susceptibility testing of anaerobes. The document suggests that susceptibility testing should be performed when anaerobes are recovered from the following infections:

- Brain abscess
- Endocarditis
- Infection of a prosthetic device or vascular graft
- Joint infection
- Osteomyelitis
- Bacteremia

Because many anaerobe-associated infectious processes are polymicrobial, deciding which anaerobic isolates warrant susceptibility testing is frequently difficult. The CLSI suggests that the following anaerobes, recognized as highly virulent and/or commonly resistant to antimicrobial agents, should be considered for testing:

- *Bacteroides fragilis* group
- *Prevotella*
- *Clostridium*
- *Fusobacterium*
- *Bilophila wadsworthia*
- *Sutterella wadsworthensis*

The CLSI working group suggests that larger laboratories periodically test several of the anaerobes listed here to determine whether the emergence of resistance to certain antimicrobial agents has occurred at their institution. Similarly, if a new antimicrobial agent has been added to the hospital formulary, testing should be performed to determine the agent's range of activity. The microbiology laboratory's decision as to which antimicrobial agents to test should be made after consultation with the hospital's infectious disease practitioners, pharmacists, and antimicrobial stewardship committee. [Table 22.19](#) lists the CLSI recommendations for the antimicrobial agents that should be considered for testing.

Problems in susceptibility testing of anaerobic isolates

Several problems have limited the routine susceptibility testing of anaerobes, including a lack of reproducibility, failure of some anaerobes to grow on or in particular media, difficulty in reading end points with certain methods, and a lack of comparability among methods. Cost and procedure complexity are other factors that inhibit laboratories from performing susceptibility testing of anaerobes.

Susceptibility testing options

CLSI document M11-A8 describes only two methods for susceptibility testing of anaerobes: agar dilution and broth microdilution. Although it is considered the gold standard method, agar dilution is labor-intensive and is not practical for use in smaller clinical laboratories. It is used primarily in reference laboratories and institutions that test large numbers of isolates. Broth microdilution panels are commercially available and can be stored frozen or lyophilized for extended periods. However, the CLSI M11-A8 document suggests that, at present, this method be used for testing members only of the *B. fragilis* group.

The Etest (AB Biodisk, Solna, Sweden) method, described in [Chapter 13](#), is an alternative method for anaerobic susceptibility testing commonly used in clinical microbiology laboratories. Testing can be performed on Mueller-Hinton agar supplemented with 5% sheep red blood cells, and plates can be incubated in anaerobe bags, jars, or chambers. Testing is limited to six antimicrobial agents on one agar plate, which is usually sufficient for most clinical situations. One critical aspect of the Etest procedure is limiting exposure to oxygen during setup and quickly achieving anaerobiasis. This is because the commonly used antimicrobial agent metronidazole is very oxygen sensitive.

Many laboratories amend their anaerobic culture reports with statements suggesting empiric agents that continue to have broad anaerobic activity. These include the carbapenems (e.g., ertapenem, imipenem, meropenem), metronidazole,

Table 22.19 Suggested antimicrobial susceptibility testing for anaerobic bacteria

	Gram-negative anaerobes (including <i>Bacteroides fragilis</i> group)	Gram-positive anaerobes
Primary choices	β -Lactam combination agent (e.g., ampicillin-sulbactam, amoxicillin-clavulanate piperacillin-tazobactam) Clindamycin Carbapenem (e.g., ertapenem, imipenem, meropenem) Metronidazole	Ampicillin or penicillin β -Lactam combination agent (e.g., ampicillin-sulbactam, amoxicillin-clavulanate, piperacillin-tazobactam) Clindamycin Carbapenem (e.g., ertapenem, imipenem, meropenem) Metronidazole
Supplemental choices	Ceftizoxime, ceftriaxone Cefoxitin Moxifloxacin	Cefoxitin, ceftizoxime, ceftriaxone Tetracycline Moxifloxacin

Modified from Clinical and Laboratory Standards Institute. (2012). *Methods for antimicrobial susceptibility testing of anaerobic bacteria: approved standard (8th ed.)*. CLSI document M11-A8. Wayne, PA: Clinical and Laboratory Standards Institute.

and the β -lactam combination antibiotics (ampicillin-sulbactam, amoxicillin-clavulanic acid, piperacillin-tazobactam). This provides health care providers with valuable information about the empiric treatment of anaerobic infections.

Treatment of anaerobe-associated diseases

There are essentially five approaches to the management of anaerobic infections.

Surgical therapy

Because most anaerobic infections are abscesses caused by mixtures of aerobic and anaerobic bacteria, the most important therapy is surgical drainage of the abscess. In less serious infections, this may be the only therapy required. Other surgical procedures include removing dead tissue (debridement), eliminating obstructions, decompressing tissues, releasing trapped gas, improving tissue circulation, and improving tissue oxygenation.

Hyperbaric oxygen

The use of a hyperbaric oxygen chamber to force oxygen into necrotic tissue has been used as adjunct therapy for anaerobic infections for many years and has gained popularity in specialty wound care clinics. Hyperbaric oxygen has also been suggested to be useful therapy for cases of osteomyelitis caused by anaerobes.

Antimicrobial therapy

The primary role of antimicrobial agents is to limit the local and systemic spread of the organisms. Selection of the correct antimicrobial agent depends on a number of factors, including types of organisms involved, known resistance factors, site of the infection, and toxicity of the antimicrobial agent. Although antimicrobial resistance has been observed with almost every agent known to have anaerobic coverage, the

carbapenems, metronidazole, and the β -lactam combination antibiotics continue to have activity against most anaerobes and are used as empiric therapy. Antimicrobial treatment of necrotic abscess is difficult because of poor circulation to the infected site.

Antitoxins

In cases of tetanus and botulism, antitoxins are used to neutralize the effect of neurotoxins produced by *C. tetani* and *C. botulinum*, respectively. Antitoxins are effective against toxin molecules that have not yet bound to host cell receptors.

Fecal microbiota transplant

Recently, refractory CDAD has been treated with replacement of the fecal microbiota. Donor feces can be obtained from a family member or purchased from a fecal transplant bank (OpenBiome, Cambridge, MA) and instilled either through colonoscopy or endoscopy. Although the technique sounds unpleasant, the cure rate of fecal microbiota transplant is over 90% in patients who have undergone numerous treatments with antimicrobials.

Case check 22.4

In the Case in Point, the patient was scheduled for immediate surgical debridement and given broad-spectrum antimicrobial therapy pending culture results. Without proper aggressive therapy, gas gangrene might require amputation. The bacteria can quickly spread, producing a fatal septicemia.

POINTS TO REMEMBER

- Varying amount of oxygen tolerance characterizes microaerophilic and facultative anaerobes and aerotolerant anaerobes, while obligate anaerobes require oxygen-free growth conditions.
- Obligate anaerobes are important in human and veterinary medicine because they can produce serious and often fatal infections and intoxications.

- Aspirates and tissue from sterile sites are more appropriate for anaerobic culture than swabs.
- Anaerobic infections of exogenous origin are usually caused by gram-positive, spore-forming bacilli belonging to the genus *Clostridium*.
- The anaerobes most frequently isolated from infectious processes in humans are those of endogenous origin, such as members of the *B. fragilis* group.
- Factors that commonly predispose the human body to anaerobic infections include trauma of mucous membranes or skin, vascular stasis, tissue necrosis, and decrease in the redox potential of tissue.
- Ports of entry for endogenous anaerobes include mucosal surfaces of oral cavities, GI tract, and GU tract.
- Significant clinical infections caused by anaerobes include mixed microbiota abscesses, tetanus, botulism, myonecrosis, CDAD, and actinomycosis.
- Many types of specimens are unacceptable for anaerobic bacteriology because they are likely to be contaminated with anaerobes of the endogenous microbiota.
- Proper selection, collection, and transport of specimens for anaerobic culture are critical for quality results and maximum recovery of pathogens.
- The anaerobes most commonly associated with infectious processes and those most often isolated from specimens include members of the *B. fragilis* group, certain *Clostridium* spp. (e.g., *C. perfringens*, *C. ramosum*, *C. clostridioforme*, *C. septicum*), *Clostridioides difficile*, *Fusobacterium nucleatum*, *F. necrophorum*, *Cutibacterium acnes*, *Actinomyces israelii*, *Porphyromonas*, *Prevotella*, and the anaerobic gram-positive cocci.
- Presumptive identifications are based on Gram stain reaction and morphology, colony appearance on different media, and results of simple, inexpensive, and rapid test procedures.
- Definitive identifications are commonly made using preformed enzyme-based identification kits, 16S rDNA sequencing, or MALDI-TOF mass spectrometry.
- Susceptibility testing of anaerobes is not recommended for all anaerobic isolates but is warranted for specific types of serious infections and whenever especially virulent or drug-resistant anaerobes have been isolated.
- Recommended methods for anaerobe susceptibility testing include microwell broth dilution panels and the Etest.
- Anaerobe-associated diseases may be treated by surgery, hyperbaric oxygen therapy, antitoxins, and antimicrobial therapy. Refractory CDAD can be treated with fecal microbiota transplant.

LEARNING ASSESSMENT QUESTIONS

1. Match the following infectious diseases with their associated causative organism:
 - _____ Myonecrosis
 - _____ Tetanus
 - _____ Botulism
 - _____ Pseudomembranous colitis
 - _____ Actinomycosis
 - a. *Clostridioides difficile*
 - b. *Clostridium perfringens*
 - c. *Clostridium tetani*
 - d. *Clostridium botulinum*

- e. *Actinomyces* spp.
2. Which organism can live in reduced concentrations of oxygen but prefers an anaerobic environment?
 - a. Capnophile
 - b. Obligate anaerobe
 - c. Facultative anaerobe
 - d. Aerotolerant anaerobe
3. Some anaerobes are particularly susceptible to oxygen because they lack which enzyme?
 - a. Amylase
 - b. β -Lactamase
 - c. Superoxide dismutase
 - d. Glucose-6-phosphate dehydrogenase
4. Which endogenous anaerobes are least likely to be involved in cases of bacteremia?
 - a. *Bacteroides*
 - b. *Clostridium*
 - c. *Eubacterium*
 - d. *Fusobacterium*
5. Which of the following specimens would be unacceptable for anaerobic culture?
 - a. Aspirated pus
 - b. Cerebrospinal fluid
 - c. Tissue from biopsy
 - d. Urethral swab
6. A gram-positive anaerobic bacillus was isolated from a wound specimen and had the following characteristics: double zone of β -hemolysis on sheep blood agar, lecithinase positive, lipase negative, spot indole negative. What is the most likely identification of this organism?
 - a. *Clostridium perfringens*
 - b. *Clostridium ramosum*
 - c. *Clostridium septicum*
 - d. *Clostridium tetani*
7. An anaerobic, pleomorphic, gram-negative bacillus was recovered from a liver abscess. The special potency antimicrobial disks demonstrated that the organism was vancomycin resistant and colistin and kanamycin sensitive. Other results were as follows: chartreuse fluorescence, spot indole positive, and lipase positive. What is the most likely identification of the organism?
 - a. *Fusobacterium mortiferum*
 - b. *Fusobacterium necrophorum*
 - c. *Fusobacterium nucleatum*
 - d. *Fusobacterium varium*

Indicate whether the following statements are true or false:

- _____ 8. Exogenous anaerobes more commonly cause infectious diseases than endogenous anaerobes.
- _____ 9. *Clostridium* spp. are especially easy to identify in Gram-stained smears of clinical specimens because they always appear as gram-positive rods with terminal or subterminal spores.
- _____ 10. Failure to isolate fusiform gram-negative organisms that were observed on a Gram-stained smear of a clinical specimen could indicate that a problem exists with the primary medium used for the isolation of anaerobes or the system being used for anaerobic incubation of primary plates.
- _____ 11. Large, dark colonies (>1 mm) growing on a BBE agar plate at 24 hours can presumptively be called a member of the *Bacteroides fragilis* group.

12. A pleomorphic gram-positive bacillus that is spot indole and catalase positive can be presumptively identified as *Cutibacterium acnes*.
13. Gram-stained smears made from a transtracheal aspirate show gram-positive branching filamentous bacilli. The culture grows colonies on anaerobic Brucella blood agar after 7 days and in thioglycollate broth after 5 days of incubation. Based on these findings, which of the following should be suspected?
- Bacteroides fragilis* group
 - Clostridium*
 - Fusobacterium*
 - Actinomyces* sp.
14. Which of the following organisms is an obligate anaerobic, gram-negative coccus?
- Bacteroides*
 - Veillonella*
 - Peptostreptococcus*
 - Fusobacterium*
15. Which of the following anaerobes is most often associated with antimicrobial resistance?
- B. fragilis* group
 - Clostridium*
 - Bacteroides* sp., not *B. fragilis*
 - Actinomyces* sp.
16. Which of the following is/are indication(s) of the presence of anaerobes?
- A double zone of hemolysis suggestive of *C. perfringens* on sheep blood agar (SBA)
 - A triple zone of hemolysis on SBA incubated anaerobically, suggestive of *C. perfringens*
 - A foul odor on opening the anaerobic jar or bag caused by metabolic end products
 - a and c
17. Anaerobic isolates can be definitively isolated via which method?
- MALDI-TOF mass spectrometry
 - Preformed enzyme-based systems
 - Biochemical-based multitest systems
 - All of the above
18. A 76-year-old male patient in a nursing home is recovering from bacterial pneumonia. The patient has been on an antimicrobial agent regimen for the past 10 days. Subsequently, he exhibits gastrointestinal symptoms consistent with pseudomembranous colitis. Which of the following organisms should be suspected?
- Clostridium perfringens*
 - Clostridioides difficile*
 - Clostridium botulinum*
 - Bifidobacterium* spp.
19. Material from an intestinal abscess produces gray colonies with a brown color in the area around the colonies on a BBE plate incubated anaerobically. There is also a dark precipitate in the medium in the areas of heavy growth. A Gram stain of the colonies reveals gram-negative coccobacilli. What is the presumptive identification of this organism?
- Bacteroides fragilis* group
 - Bacteroides vulgatus*
 - Bacteroides denticola*
 - Peptostreptococcus* sp.
20. An immunosuppressed patient notices sinus tracts that are draining pus. He also notices that there appear to be small, hard "nuggets" in the pus. What disease is he most likely suffering from?
- Gas gangrene
 - Pseudomembranous colitis
 - Actinomycosis
 - Myonecrosis

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The spirochetes

Amy M. Woron and A. Christian Whelen

CHAPTER OUTLINE

Leptospira, 534

- General characteristics, 534
- Virulence factors and pathogenicity, 534
- Infections caused by leptospires, 534
- Epidemiology, 535
- Laboratory diagnosis, 535
- Antimicrobial susceptibility, 536
- Borrelia*, 536
- Borrelia recurrentis* and similar borreliae, 536
- Borrelia burgdorferi* sensu lato, 537

Treponemes, 538

- General characteristics, 538
- Clinically significant species, 538
- Treponema pallidum* subsp. *pallidum*, 538
- Other treponemal diseases, 541

Bibliography, 542

OBJECTIVES

After reading and studying this chapter, you should be able to:

1. Describe the general characteristics of the genera of spirochetes.
2. Discuss the risk factors associated with relapsing fever infection.
3. Describe the pathogenesis and clinical manifestations of the borrelioses.
4. Compare the causative agents and arthropod vectors of relapsing fever and Lyme borreliosis.
5. Describe the laboratory diagnosis of relapsing fever and how it differs from the diagnosis of other spirochetal diseases in the United States.
6. Justify the use of the two-tiered approach to laboratory diagnosis of Lyme borreliosis.
7. Compare the four human pathogens of the genus *Treponema*.
8. Describe the clinical manifestations of the primary, secondary, and tertiary stages of syphilis.
9. Discuss the epidemiology of leptospirosis in the United States.
10. Evaluate the tests used to diagnose *Treponema pallidum* infections in the clinical laboratory.

KEY TERMS

- | | |
|-----------------------------|--|
| Chancere | Rapid plasma reagin (RPR) test |
| Endemic relapsing fever | Relapsing fever |
| Endemic syphilis | Spirochetes |
| Epidemic relapsing fever | Syphilis |
| Erythema migrans (EM) | Venereal Disease Research Laboratory (VDRL) test |
| Gummas | Weil disease |
| Jarisch-Herxheimer reaction | Yaws |
| Leptospirosis | Zoonoses |
| Lyme borreliosis | |
| Pinta | |

Case in point

A 29-year-old male patient arrived at a local medical clinic in Los Angeles complaining of diarrhea, fever, chills, muscle aches, and headaches. He had returned 2 days earlier after competing in the Eco-Challenge in Malaysian Borneo. During the competition, he completed various events, including mountain biking, caving, climbing, jungle trekking, swimming, and kayaking in freshwater and salt water. He was still recovering from multiple abrasions from the jungle trekking and mountain biking. While kayaking on the Segama River, his kayak capsized, and he inadvertently swallowed several mouthfuls of river water. His two teammates took doxycycline as malaria prophylaxis before and during the race. Neither of them became ill.

Issues to consider

After reading the patient's case history, consider:

- Risk factors for acquiring infectious disease for the patient
- Spirochete agents that cause influenza-like illness and methods to identify or rule out those agents
- Effective prophylaxis, if available, for influenza-like illness caused by spirochetes
- Empiric therapy options

The class Spirochaetia contains four orders: Brachyspirales, Brevinematales, Leptospirales, and Spirochaetales. The order Leptospirales contains the family Leptospiraceae, and the order Spirochaetales contains four families: Borreliaceae, Sphaerochaetaceae, Spirochaetaceae, and Treponemataceae. The family Leptospiraceae contains the genus *Leptospira*, the family Borreliaceae contains the genus *Borrelia*, and the family Treponemataceae contains the genus *Treponema*. These three genera include the causative agents of important human diseases, such as **syphilis** (sexually transmitted); **leptospirosis**, a **zoonoses** (transmitted from animals to humans); and Lyme borreliosis (or Lyme disease) and **relapsing fever**, both of which are vector-borne diseases. There has been a recent resurgence in primary and secondary syphilis as well as the severe disease congenital syphilis in the United States. Leptospirosis is likely a prevalent, underreported disease found worldwide.

Spirochetes are slender, flexuous, helically shaped, unicellular bacteria ranging in size from 0.1 to 0.5 μm wide and from 5 to 20 μm long, with one or more complete turns in the helix. They differ from other bacteria in that they have a flexible cell wall around which several fibrils are wound. These fibrils, termed *periplasmic flagella* (also known as *axial fibrils*, *axial filaments*, *endoflagella*, and *periplasmic fibrils*), are responsible for motility. A multilayered outer sheath resembling the outer membrane of gram-negative bacteria surrounds completely the protoplasmic cylinder (the cytoplasmic and nuclear regions are enclosed by the cytoplasmic membrane-cell wall complex and periplasmic flagella). The spirochetes exhibit various types of motion in liquid media.

Spirochetes are free living or survive in association with animal and human hosts as normal biota or pathogens. In addition, they can use carbohydrates, amino acids, long-chain fatty acids, or long-chain fatty alcohols as carbon and energy sources. Metabolism can be anaerobic, facultatively anaerobic, or aerobic, depending on the species. *Treponema* spp. reproduce via transverse fission, whereas *Leptospira* and *Borrelia* divide by the more common binary fission.

Leptospira

General characteristics

Historically, pathogenic leptospire were identified as *Leptospira interrogans*, and environmental saprophytes were named *Leptospira biflexa*. Currently, the leptospire are divided into serovars (serotypes). More than 200 different serovars of *L. interrogans* sensu lato are recognized. Antigenically related serovars are placed into serogroups, which have no taxonomic standing but are useful for epidemiologic studies. Genetic typing (16S rRNA gene) based on nucleic acid similarities has identified 23 genomospecies that include all of the *Leptospira* serovars. Although genetic typing is taxonomically correct, scientists and health care providers continue to prefer serogroup-based nomenclature. Identification requires determining serovars by serotyping and determining species by nucleic acid sequencing or matrix-assisted laser desorption/ionization–time-of-flight (MALDI-TOF) mass spectrometry.

Organisms of the genus *Leptospira* are tightly coiled, thin, flexible spirochetes, 0.1 μm wide and 5 to 15 μm long (Fig. 23.1).



Fig. 23.1 Dark-field image of *Leptospira interrogans* serotype Sejroe Wolffii 3705. The tight coils and bent ends are characteristic of this organism ($\times 1000$). (Courtesy State Laboratories Division, Hawaii Department of Health.)

In contrast with both *Treponema* and *Borrelia* organisms, the spirals are very close together, so the organism may appear to be a chain of cocci. One or both ends of the organism have hooks rather than tapering off. Their motion is rapid translational (back and forth) and rotational.

Electron microscopy reveals a long axial filament covered by a very fine sheath, like treponemes and borreliae. All species have two periplasmic flagella. The organisms cannot be readily stained, but they can be impregnated with silver and visualized. Unstained cells are not visible by bright-field microscopy but are visible by dark-field, phase-contrast, and immunofluorescent microscopy. Leptospire are obligate aerobes.

Virulence factors and pathogenicity

Leptospiral disease in the United States is caused by more than 20 different serovars, the most common of which are Icterohaemorrhagiae, Australis, and Canicola. Some serovars of *L. interrogans* sensu lato and *L. biflexa* sensu lato are pathogenic for a wide range of wild and domestic animals and humans, but the mechanisms of pathogenicity are not well understood. Factors that may play a role in pathogenicity include reduced phagocytosis in the host, a soluble hemolysin produced by some virulent strains, cell-mediated sensitivity to leptospiral antigen by the host, and small amounts of endotoxins produced by some strains. The clinical findings in animals with leptospirosis suggest the presence of endotoxemia.

Infections caused by leptospire

Zoonotic leptospire contaminate water or mud when shed in the urine of infected animals and often enter the human host through small breaks in the skin or intact mucosa. The initial sites of multiplication are unknown. Nonspecific host defenses do not stop multiplication of leptospire, and

leptospiremia occurs during acute illness. Late manifestations of the disease can be caused by the host's immunologic response to the infection.

The incubation period of leptospirosis is usually 10 to 12 days but ranges from 3 to 30 days. Individuals often do not seek treatment because the majority of cases are subclinical or very mild. The clinical presentation is usually abrupt, with nonspecific, influenza-like symptoms, such as fever, chills, headache, severe myalgia, and malaise. The subsequent course is protean, frequently biphasic, and can result in hepatic, renal, and central nervous system (CNS) involvement. The major renal lesion is an interstitial nephritis with associated glomerular swelling and hyperplasia that does not affect the glomeruli. The most characteristic physical finding is conjunctival suffusion, but this is seen in less than 50% of patients. Severe systemic disease (**Weil disease**) occurs in about 5% to 10% of cases and includes renal failure, hepatic failure, and intravascular disease and can result in death. The duration of the illness ranges from less than 1 week to 3 weeks. Late manifestations can be caused by the host's immunologic response to the infection. In patients with a leptospiral bacteremia, immunoglobulin M (IgM) antibodies are detected within 1 week after onset of disease and can persist in high titers for many months. Immunoglobulin G (IgG) antibodies are usually detectable 1 month or more after infection. Convalescent serum contains protective antibodies.

Epidemiology

Leptospira can be free living or associated with colonization of kidneys in animals. The most important hosts are small mammals. Worldwide, leptospirosis is considered the most common zoonosis, causing an estimated over 1 million cases annually. In 2013, leptospirosis was reinstated as a notifiable disease in the United States. The Centers for Disease Control and Prevention (CDC) estimates that 100 to 150 cases of leptospirosis occur annually in the United States, mainly in Puerto Rico, followed by Hawaii. Since most cases are believed to be asymptomatic, cases likely remain unrecognized nationwide and go unreported.

Leptospirosis is a zoonosis primarily associated with occupational or recreational exposure. Working with animals or in rat-infested surroundings poses hazards for veterinarians, dairy workers, swine handlers, slaughterhouse workers, miners, sewer workers, and fish and poultry processors. In the United States, most cases of leptospirosis result from recreational exposures.

In the natural host, leptospires live in the lumen of renal tubules and are excreted in urine. Dogs, rats, and other rodents are the principal animal reservoirs. Hosts acquire infections directly by contact with the urine of carriers or indirectly by contact with bodies of water contaminated with the urine of carriers. Leptospires can survive in neutral or slightly alkaline waters for months. Protective clothing (boots and gloves) should be worn in situations involving possible occupational exposure to leptospires. Control measures include rodent elimination and drainage of contaminated waters. Vaccination of dogs and livestock has been effective in preventing disease but not the initial infection and leptospiuria. Short-term prophylaxis consisting of weekly doxycycline therapy may be appropriate in high-risk groups with expected occupational exposure.

Case check 23.1

Leptospires are present in water and mud contaminated by the urine of reservoir animals. The Case in Point describes significant and repeated exposure risk that should be reported to the primary health care provider on presentation. Otherwise, the initial clinical impression might resemble influenza, especially if presentation occurs during periods of high influenza activity.

Laboratory diagnosis

Specimen collection and handling

Leptospiremia occurs during the acute phase (first week) of the disease, before symptoms are present. Toward the end of the first week as symptoms appear (first 4 to 6 days of illness), blood or cerebrospinal fluid (CSF) should be collected. Optimal recovery occurs if fresh specimens are inoculated directly into laboratory media. Urine can also be collected, but the yield is much higher after the first week of illness, and shedding can occur intermittently for weeks. Ideal submission is whole blood and serum during the first week of acute illness and serum with optional urine during convalescent illness.

Microscopic examination

Although direct demonstration of leptospires in clinical specimens during the first week of the disease by special stains, dark-field microscopy, or phase-contrast microscopy is possible, it is not recommended. Direct demonstration is successful in only a small percentage of cases, and false-positive results may be reported because of the presence of artifacts, especially in urine.

Isolation and identification

Isolation of leptospires is accomplished by direct inoculation of one to two drops of freshly drawn blood or CSF into laboratory media, such as Fletcher semi-solid medium, Stuart liquid medium, or Ellinghausen-McCullough-Johnson-Harris (EMJH) semi-solid medium, and incubation of the media in the dark at room temperature. Several dilutions of urine should be used (undiluted, 1:10, and 1:100) and/or filtered (0.45 μ m) to minimize the effects of inhibitory substances. Tubes are examined weekly for evidence of growth, such as turbidity, haze, or a ring of growth. A drop taken from a few millimeters below the surface is examined by dark-field microscopy for tightly coiled, rapidly motile spirochetes with hooked ends. Serotypes have historically been identified by microscopic agglutination testing using sera of defined reactivity; however, 16S ribosomal nucleic acid sequencing and MALDI-TOF mass spectrometry have been shown to provide accurate species identification.

Polymerase chain reaction (PCR) testing for leptospire DNA in blood, plasma, serum, urine, CSF, and tissue is as sensitive or more so than culture methods. PCR has the advantage of providing a diagnosis in the acute stage, when treatment is most beneficial. Real-time PCR can also quantify the infection burden.

Serologic tests

Most cases of leptospire infection are diagnosed by serology. In patients with leptospiral bacteremia, IgM antibodies are detected within 1 week after onset of disease and may persist in high titers for many months. One month or more after the onset of illness, IgG antibodies can be detected in some patients. A visually read IgM enzyme-linked immunosorbent assay (ImmunoDOT *Leptospira* IgM; GenBio, San Diego, CA) has been approved by the U.S. Food and Drug Administration (FDA) and has demonstrated good performance in cases of acute leptospirosis. A macroscopic slide agglutination test for rapid screening and the gold standard microscopic agglutination test are available for detection of leptospiral antibodies, but both require maintenance of defined serotypes in culture, so their use is typically limited to confirmatory laboratories. Acute and convalescent samples are required to demonstrate a fourfold rise in titer to confirm a diagnosis.

Antimicrobial susceptibility

Susceptibility testing of leptospires is not normally performed in the clinical laboratory; leptospires have been shown to be susceptible in vitro to streptomycin, tetracycline, doxycycline, and the macrolide antimicrobials. For severe disease, intravenous penicillin and ceftriaxone are equally effective. Doxycycline appears to shorten the course of the illness in adults and reduce the incidence of convalescent leptospiruria.

Case check 23.2

At least two deaths occurred in 2009, when confusion with pandemic influenza delayed appropriate antimicrobial therapy in patients with severe leptospirosis. The Case in Point describes two teammates who were taking doxycycline for malaria prophylaxis, which is also effective against many bacterial agents, including *Leptospira*. Adherence to this preventive medicine regimen likely contributed to disease avoidance in these individuals.

Borrelia

General characteristics

The genus *Borrelia* comprises several species of spirochetes that are morphologically similar but have different pathogenic properties and host ranges. Some species cause relapsing fever, whereas several other species cause **Lyme borreliosis**. All pathogenic *Borrelia* are arthropod-borne. Over 20 species are categorized as relapsing fever *Borrelia* that include *Borrelia recurrentis*, *Borrelia hermsii*, and *Borrelia duttonii*. The *B. burgdorferi* sensu lato complex, sometimes simply called *B. burgdorferi*, causes a spectrum of syndromes known as Lyme borreliosis or Lyme disease. Over 20 species belong to this group and include *B. burgdorferi* sensu stricto (often referred to as *B. burgdorferi*), *Borrelia afzelii*, *Borrelia bavariensis*, and *Borrelia bissettii*.

Borreliae are highly flexible organisms ranging in thickness from 0.2 to 0.5 μm and in length from 3 to 20 μm . The spirals range in number from 3 to 10 per cell and are much less tightly coiled than those of the leptospires (Fig. 23.2). Unlike

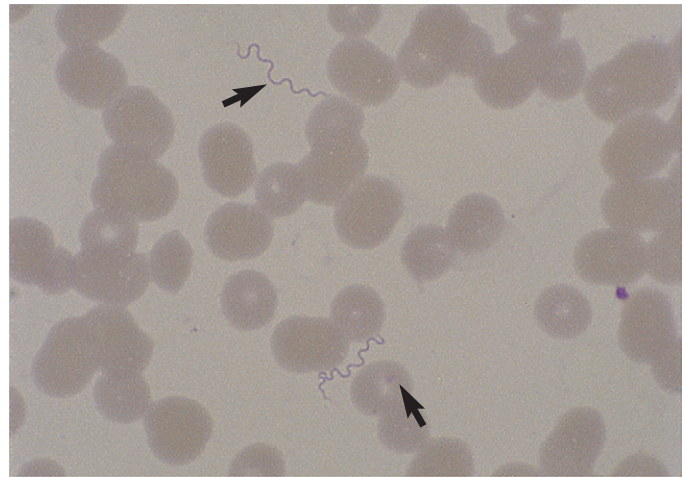


Fig. 23.2 Appearance of *Borrelia recurrentis* (arrows) in blood (Giemsa stain, $\times 850$).

leptospires and treponemes, borreliae stain easily and can be visualized by bright-field microscopy. Electron microscopy shows the same general features as are seen with the treponemes—long, periplasmic flagella (15 to 20 per cell) coated with sheaths of protoplasm and periplasm.

Borrelia recurrentis and similar borreliae

Virulence factors

As the disease name suggests, relapsing fever is characterized by acute febrile episodes that subside spontaneously but tend to recur over a period of weeks. *Borrelia* spp. responsible for this disease first evade complement by acquiring and displaying suppressive complement regulators, C4b-binding protein, and factor H. The relapses are potentiated by antigenic variation; borreliae systematically change their surface antigens, thereby rendering specific antibody production ineffective in completely clearing the organisms.

Clinical manifestations

After an incubation period of 2 to 15 days, massive spirochetemia develops and remains at varying levels of severity during the entire course of relapsing fever. The infection is accompanied by sudden high temperature, rigors, severe headache, myalgia, arthralgia, and weakness. The febrile period lasts about 3 to 7 days and ends abruptly with the development of an adequate immune response. The disease recurs several days to weeks later, following a less severe but similar course. The febrile periods worsen during the spirochetemia and wane as the immune response clears the bacteria from circulation.

Epidemiology

Relapsing fever can be tickborne (**endemic relapsing fever**) or louseborne (**epidemic relapsing fever**). Tickborne borreliae are transmitted by a large variety of soft ticks of the genus

Ornithodoros except for *Borrelia miyamotoi*, which is believed to be transmitted by the larval hard tick *Ixodes scapularis*. Tickborne borreliae are widely distributed throughout the world, although specific species of borreliae tend to be limited geographically by their vector. Transmission to a vertebrate host takes place via infected saliva during tick attachment.

Louseborne fever, due to *B. recurrentis*, is transmitted via the body louse, *Pediculus humanus*, and humans are the only reservoir. Borreliae infect the hemolymph of the louse. Unlike tickborne disease, transmission of the louseborne disease occurs when infected lice are crushed and scratched into skin, rather than through the bite of an infected arthropod. Relapsing fever is best prevented by controlling exposure to the arthropod vectors. For tickborne relapsing fever, limiting exposure to ticks includes wearing protective clothing, rodent control, and the use of insect repellents. For louseborne relapsing fever, control is best achieved by good personal and public hygiene, especially improvements in measures to avoid overcrowding and in delousing.

Laboratory diagnosis

Microscopic examination

Diagnosis of borreliosis is readily made by observing Giemsa- or Wright-stained smears of peripheral blood taken during the febrile period collected in ethylenediaminetetraacetic acid (EDTA). Relapsing fever is the only spirochetal disease in which the organisms are visible in blood with bright-field microscopy. The appearance of the spirochete among the red blood cells is characteristic (see Fig. 23.2).

Isolation and identification

Whole blood collected in EDTA can also be used for culture and PCR testing, although citrate-treated blood might be superior. Borreliae can be recovered by using Barbour-Stoenner-Kelly medium, but it is rarely attempted. *B. recurrentis*, *Borrelia hermsii*, *Borrelia parkeri*, *Borrelia turicatae*, and *Borrelia hispanica* have been successfully cultivated. Antigenic variation in the spirochetes that cause relapsing fever makes the serodiagnosis of their diseases difficult and impractical with the possible exception of *B. miyamotoi*. Because the borrelia can be readily detected microscopically and by PCR, serologic methods have limited use.

Antimicrobial susceptibility

Borreliae are susceptible to many antimicrobial agents; however, tetracyclines are the drugs of choice because they reduce the relapse rate and rid the CNS of spirochetes. Up to 39% of patients treated with antimicrobial agents experience fever, chills, headache, and myalgia believed to be caused by the sudden release of endotoxin from the spirochetes, a condition referred to as **Jarisch-Herxheimer reaction**.

Borrelia burgdorferi sensu lato

Virulence factors

Bacterial spread may occur depending on the organism's ability to bind plasminogen and urokinase-type plasminogen

activator to its surface. This binding can convert plasminogen to plasmin, which is a potent protease and could facilitate tissue invasion. Binding factor H allows complement evasion and immune system suppression and might explain, in part, why IgM antibody titer does not peak for 3 to 6 weeks. In vitro, the organism can stimulate proinflammatory cytokines, such as tumor necrosis factor and interferons, which can be important in controlling disease but may also contribute to inflammatory manifestations as untreated disease progresses.

Clinical manifestations

Lyme borreliosis is a complex disease that can generally be divided into three stages. Early infection includes two stages, the first of which is localized (stage 1). About 60% to 80% of patients exhibit **erythema migrans (EM)**, the classic skin lesion that is normally found at the site of the tick bite, within 1 week of the tick bite. It begins as a red macule and expands to form large annular erythema with partial central clearing, sometimes described as having a target ("bull's eye") appearance. EM often occurs alone but can be accompanied by fever, fatigue, headache, mild stiff neck, arthralgia, or myalgia. Stage 2 is disseminated early and produces widely variable symptoms that include secondary skin lesions, migratory joint and bone pain, alarming neurologic and cardiac disease, splenomegaly, and severe malaise and fatigue. Late manifestations, or late persistent infections (stage 3), occur mainly in the cardiac, musculoskeletal, and neurologic systems. Arthritis is the most common symptom, occurring weeks to years later.

Epidemiology

Lyme borreliosis is the number one vector-borne disease in the Northern Hemisphere. Organisms are transmitted via the bite of infected *Ixodes* ticks, so most cases occur from June to September, when more people are involved in outdoor activities and ticks are more active. Lyme disease was first described after an outbreak among children in Lyme, Connecticut, in 1975. A total of 23,453 confirmed and 11,492 probable cases were reported in the United States in 2019. The CDC estimates as many as 300,000 Americans contract Lyme borreliosis annually.

At least three genospecies of *B. burgdorferi* sensu lato cause Lyme borreliosis. *B. burgdorferi*, *B. garinii*, and *B. afzelii* are the most common causes of Lyme borreliosis in the United States and Europe. Protective clothing and repellents should be used in areas in which tick exposure is intense. Attached ticks should be removed immediately because pathogen transmission is associated with the length of attachment.

Laboratory diagnosis

Specimen collection and handling

The most common and productive specimen for the laboratory diagnosis of *B. burgdorferi* sensu lato infection is serum for serology. Other tests have too many limitations (e.g., polymerase chain reaction) or have been inadequately validated (e.g., urine and CSF antigen). Large-volume plasma cultures have been shown to be positive in about 50% of adult patients

with erythema migrans. The paucity of bacteria present in clinical samples renders direct microscopy insensitive.

Serologic tests

Diagnosis follows a two-tier approach in which the first step is a sensitive immunofluorescence antibody (IFA), chemiluminescence immunoassay (CLIA), or enzyme immunoassay (EIA) screening that detects IgM and IgG. If the test result is negative, no further testing is needed. Reactive or equivocal results are confirmed with separate IgM and IgG Western immunoblots. If symptoms were present for 30 days or less, the patient is tested for IgM only. Immunoblot confirmation of IgM antibody presence includes reactivity for two of the three following bands: 24, 39, and 41 kilodaltons (kDa). Confirmation of IgG antibody presence is acceptable when 5 of the 10 scored bands are present: 18, 21, 28, 30, 39, 41, 45, 58, 66, and 93 kDa. Because of the low sensitivity of the immunoblot, two-tiered testing is insensitive, particularly in patients with early disease.

To improve sensitivity, in 2019, the FDA cleared several serologic tests that use EIA and CLIA rather than immunoblot in a modified two-test approach. These assays detect antibodies to the proteins C6 and VlsE found in *B. burgdorferi*. If serologic test results are negative and symptoms are consistent with Lyme borreliosis, a convalescent serum should be obtained and tested.

Antimicrobial susceptibility

Early diagnosis and antimicrobial treatment are important for preventing neurologic, cardiac, and joint abnormalities that can occur late in the disease. Doxycycline is the recommended therapy, but macrolides and β -lactams have also been effective in treating early stages of Lyme disease without complications. For refractile or late stages, prolonged treatment with ceftriaxone has been effective. Antimicrobial susceptibility testing is not warranted.

Treponemes

General characteristics

Pathogenic treponemes are thin, spiral organisms about 0.1 to 0.2 μm thick and 6 to 20 μm in length. They are difficult to visualize with a bright-field microscope because they are very thin, but they can be seen easily on dark-field microscopy. The spirals are regular and angular, with 4 to 14 spirals per organism (Fig. 23.3). Three periplasmic flagella are inserted into each end of the cell. The ends are pointed and covered with a sheath. The cells exhibit graceful flexuous movements in liquid.

Clinically significant species

The genus *Treponema* comprises four microorganisms that are pathogenic for humans: *T. pallidum* subsp. *pallidum*, the causative agent of syphilis; *T. pallidum* subsp. *endemicum*, the causative agent of **endemic syphilis**; *T. pallidum* subsp. *pertenue*, the causative agent of **yaws**; and *Treponema carateum*, the

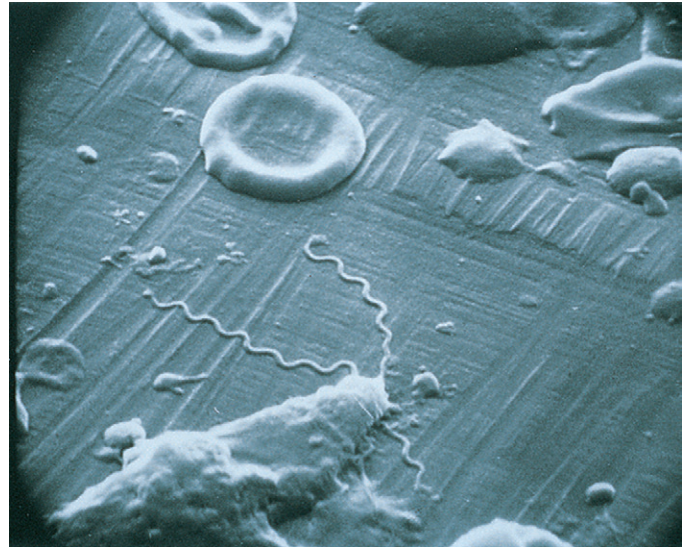


Fig. 23.3 Scanning electron micrograph of *Treponema pallidum*. Two treponemes are shown adjacent to an erythrocyte (Nichols strain, $\times 2500$).

causative agent of **pinta**. The four pathogenic strains exhibit a high degree of DNA homology ($>99\%$) and shared antigens. At least six nonpathogenic species have been identified in the normal microbiota, and they are particularly prominent in the oral cavity. Some of these species, notably *Treponema denticola*, have been linked to the polymicrobial infections gingivitis and periodontitis.

Treponema pallidum subsp. *pallidum*

Virulence factors

Treponema pallidum subsp. *pallidum* can cross intact mucous membranes and the placenta, disseminate throughout the body, and infect almost any organ system. It has also been postulated that antigenic variation of cell surface proteins contributes to the organism's ability to evade host immune response and establish persistent infection.

Clinical manifestations

Treponema pallidum subsp. *pallidum* causes syphilis. The word *syphilis* comes from a poem written in 1530 that described a mythical shepherd named Syphilus, who was afflicted with the disease as punishment for cursing the gods. The poem represented the compendium of knowledge at the time about the disease.

Treponema pallidum subsp. *pallidum* transmission normally occurs during direct sexual contact with an individual who has an active primary or secondary syphilitic lesion. Consequently, the genital organs—the vagina and cervix in females and the penis in males—are the usual sites of inoculation. Syphilis can also be acquired by nongenital contact with a lesion (e.g., on the lip) or transplacental transmission to a fetus, resulting in congenital syphilis. After bacterial invasion through a break in the epidermis or penetration through intact mucous membranes, the natural course of syphilis can be divided into primary, secondary, and tertiary stages based

on clinical manifestations. Co-infection with human immunodeficiency virus (HIV) can result in variation of the natural course of the disease. Furthermore, ulcers caused by syphilis might contribute to the efficiency of HIV transmission in populations with high rates of both infections. Syphilis has a wide variety of clinical manifestations, which gave rise to the name the “great imitator.”

Primary stage of syphilis

The three stages of syphilis are primary, secondary, and tertiary. For reporting and surveillance purposes, the CDC uses the case definition categories primary; secondary; early, nonprimary nonsecondary; and unknown duration or late latent. After inoculation, the spirochetes multiply rapidly and disseminate to local lymph nodes and other organs via the bloodstream. The primary mucocutaneous lesion develops 10 to 90 days after infection and is a result of an inflammatory response to the infection at the site of inoculation. The lesion, known as a chancre, is typically a single erythematous lesion that is nontender but firm, with a clean surface and raised border. The lesion is teeming with treponemes and is extremely infectious. Because the chancre is commonly found on the cervix or vaginal wall and is nontender, the lesion might not be obvious in women. The lesion can also be found in the anal canal of both sexes and remain undetected. The penis is the usual location of chancres in men. No systemic signs or symptoms are evident in the primary stages of the disease.

Secondary stage of syphilis

Approximately 4 to 10 weeks after development of the primary lesion, due to spirochete dissemination, the patient may experience secondary disease, with clinical symptoms of fever, sore throat, generalized lymphadenopathy, headache, lesions of the mucous membranes, and rash. The rash can present as macular, papular, follicular, papulosquamous, or pustular and is unusual in that it can also occur on the palms and soles. All secondary lesions of the skin and mucous membranes are highly infectious. The secondary stage can last for several weeks and can relapse. It might also be mild and go unnoticed by the patient. Relapses of secondary syphilis occur in about 25% of untreated patients.

Tertiary stage of syphilis

Following the secondary stage, patients enter latent syphilis, when clinical manifestations are absent, and the patient is not infectious. Latency within 1 year of infection is referred to as *early latent syphilis*, whereas latency greater than 1 year is *late latent syphilis*. Approximately one third of untreated patients exhibit biological cure, losing serologic reactivity. Another one third remain latent for life but have reactive sera. The remaining one third ultimately develop tertiary or late syphilis, generally decades later. Symptoms of tertiary syphilis include the development of granulomatous lesions (**gummas**) in skin, bones, and the liver (benign tertiary syphilis), degenerative changes in the CNS (neurosyphilis), and syphilitic cardiovascular lesions, particularly aortitis, aneurysms, and aortic valve insufficiency. Patients in the tertiary stage are usually not infectious. In the United States and most developed countries, the tertiary stage of disease is not often seen because most patients are adequately treated with antimicrobial agents before the tertiary stage is reached.

Congenital syphilis

Treponemes can be transmitted from an infected mother to her fetus by crossing the placenta. Congenital syphilis affects many body systems and is therefore severe and mutilating. Early-onset congenital syphilis (onset before 2 years of age) resembles secondary syphilis in adults. It is characterized by mucocutaneous lesions, osteochondritis, anemia, hepatosplenomegaly, and CNS involvement, and it occurs when mothers have early syphilis during pregnancy. Late-onset congenital syphilis, corresponding to tertiary syphilis in adults, results following pregnancies when mothers have chronic, untreated infections. Symptoms of late-onset congenital syphilis occur after 2 years of age but generally are not apparent until the second decade of life. Symptoms include interstitial keratitis, bone and tooth deformities, cranial nerve VIII deafness, neurosyphilis, and other tertiary manifestations.

Epidemiology

Under natural conditions, *Treponema pallidum* subsp. *pallidum* is an exclusively human pathogen. Syphilis was first recognized in Europe at the end of the 15th century, when it reached epidemic proportions. Two theories have been proposed concerning the introduction of syphilis into Europe. One theory suggests that Christopher Columbus's crew brought the disease from the West Indies back to Europe. The second theory suggests that the disease was endemic in Africa and transported to Europe via the migration of armies and civilians. The venereal transmission of syphilis was not recognized until the 18th century. The causative agent of syphilis was not discovered until 1905.

The incidence of syphilis in the United States decreased through the 1990s, and the fewest cases (31,618) since reporting began in 1941 were reached in 2000. However, since 2000, the incidence of the disease has been increasing almost every year, including a 16.4% increase from 2018 to 2020. In 2020, 133,945 cases of all stages of syphilis were reported, which included 41,655 cases of primary and secondary syphilis. From 2016 to 2022, cases of primary and secondary syphilis per 100,000 increased from 15.5 to 20.8 in males and 1.9 to 4.7 in females. Men who have sex with men account for the majority of reported primary and secondary syphilis cases (56.7% in 2019); however, heterosexual syphilis is also increasing. It is up 58.2% among women from 2018 to 2020. An increase in the rate among women corresponds to an increase in the rate of congenital syphilis. The rate of congenital syphilis increased from 8.4 per 100,000 live births in 2012 to 57.3 per 100,000 live births in 2020.

High-risk sexual behavior and co-infection with HIV continue to complicate syphilis control efforts. Educating people about sexually transmitted diseases (STDs), including providing information about the proper use of barrier contraceptives, reporting each case of syphilis to public health authorities for contact investigation, and treating all sexual contacts of persons infected with syphilis are cornerstones of syphilis control efforts. Serologic screening of high-risk populations should be performed, and to avoid congenital syphilis, pregnant women should undergo serologic testing during early and late pregnancy.

Laboratory diagnosis

Specimen collection and handling

Lesions of primary and secondary syphilis typically contain large numbers of spirochetes. The surface of primary or secondary lesions is cleansed with saline and gently abraded with dry, sterile gauze; induction of bleeding should be avoided. Serous transudate is placed onto a slide, diluting it with nonbactericidal saline if the preparation is thick. A coverslip is added, and the slide is transported immediately to a laboratory, where dark-field microscopy is performed. Oral lesions should not be examined because the numerous non-pathogenic spirochetes present in these specimens will lead to misinterpretation. Culture methods are not available, and dark-field microscopy equipment and expertise are uncommon, so serology is the normal basis of diagnosis. Nucleic acid amplification tests are not commonly used, and no FDA-cleared commercial assay is available. Rabbit inoculation tests are considered the gold standard, but they are not practical for routine diagnosis.

Microscopic examination

The organisms are too thin to be observed by bright-field microscopy, so spirochetes are illuminated against a dark background. Dark-field microscopy requires considerable skill and experience; however, demonstration of motile treponemes in material from the chancre is diagnostic for primary syphilis.

Serologic tests

Serology is the primary method used for the laboratory diagnosis of syphilis. Because of the antigenic similarity between the four *Treponema* strains pathogenic for humans, serologic assays cannot distinguish them. Two major types of serologic tests exist: nontreponemal and treponemal. Both have lower sensitivities in the primary stage of syphilis, but approach 100% in the secondary stage. Treponemal tests retain a very high sensitivity in the tertiary stage as well, whereas nontreponemal tests have a lower sensitivity. A co-infection with HIV can result in false-negative serologic test results. Comparisons between CSF and serum antibody responses can be helpful in cases of neurosyphilis. With congenital syphilis, comparing antibody responses in the mother's serum and infant's serum can aid in the diagnosis.

The nontreponemal tests detect reaginic antibodies that develop against lipids released from damaged cells. Although they are biologically nonspecific and known to react with organisms of other diseases and conditions (causing false-positive reactions), the nontreponemal tests are excellent screening tests. The antigen used is a precisely defined cardiolipin-lecithin-cholesterol complex made from bovine hearts. Nontreponemal tests have a lower specificity than treponemal tests. Because a nontreponemal antigen is used in these assays, they are prone to false-positive reactions. All reactive results should be confirmed with a treponemal antigen test.

The two nontreponemal tests widely used today are the **Venereal Disease Research Laboratory (VDRL) test** and the **rapid plasma reagin (RPR) test**. These tests are inexpensive to perform, demonstrate rising and falling reagin titers, and correlate with the clinical status of the patient. For the VDRL test, cardiolipin antigen is mixed with the patient's serum or CSF.

Flocculation is a positive reaction and is observed microscopically. The RPR test is more commonly used; carbon particles are used to visually enhance the reaction and is read on a white card macroscopically. When mixed with a positive serum on a disposable card, the black charcoal particles clump together with the cardiolipin-antibody complexes. The flocculation is easily observed without a microscope. Reactive or weakly reactive sera should undergo titration and be tested with a treponemal test. Recently automated RPR testing has gained FDA clearance. A third nontreponemal antigen test occasionally used is the toluidine red unheated serum test. It is similar to the RPR assay, except toluidine red is substituted for charcoal.

The treponemal tests detect antibodies specific for treponemal antigens. Historically, they have been used to confirm positive nontreponemal test results, although some laboratories use reverse sequence syphilis screening. In this strategy, automated treponemal test-positive serum is retested with a nontreponemal assay and a second treponemal assay. This strategy resulted in higher numbers of false-positive results in five laboratories studied from 2006 to 2010, so the CDC continues to recommend the original approach. Treponemal tests are also helpful in the detection of late-stage infections because the titers remain high and usually do not drop in response to therapy, as with nontreponemal tests. Consequently, treponemal tests are not useful in following therapy or detecting reinfection.

The treponemal antigens used are spirochetes derived from rabbit testicular lesions. Two commonly used treponemal antigen test methods are the *Treponema pallidum*-particulate agglutination (TP-PA) test (Fujirebio America, Fairfield, NJ) and EIAs. The TP-PA test uses gelatin particles sensitized with *T. pallidum* antigens. Agglutination indicates the presence of antitreponemal antibodies. EIA kits are simple to use, commercially available, and comparable with other treponemal tests. They also have the advantage of being semi-automated. The majority use recombinant *T. pallidum* antigens and detect IgG, IgM, or both. The fluorescent treponemal antibody absorption (FTA-ABS) assay uses a fluorescent-labeled antihuman antibody that detects patient antitreponemal antibodies bound to treponema affixed to a commercially prepared slide. Because of subjectivity in reading the samples and the use of expensive fluorescent microscopy, the FTA-ABS test has become less frequently used, and agglutination assays and EIAs are favored.

Neurosyphilis is diagnosed based on clinical and laboratory findings. The VDRL test on CSF has high specificity but low sensitivity. Because of plasma IgG entering the CSF, the FTA-ABS assay on CSF has low specificity but high sensitivity for neurosyphilis. Therefore a negative FTA-ABS assay result is a strong indicator against neurosyphilis, but a negative VDRL test result cannot rule out neurosyphilis.

Rapid immunochromatographic, point-of-care assays using whole blood collected via finger stick have demonstrated sensitivities and specificities like those of *Treponema* antigen tests. Reactive samples need a nontreponemal antigen titer test. The assays employ recombinant *T. pallidum* antigens to detect IgG and IgM. They are useful in STD diagnosis and antenatal clinics. Immunoblot assays are commercially available; some use fractionated *T. pallidum* proteins, and others use recombinant bacterial proteins. The assays can differentiate between IgG and IgM if a separate anti-human immunoglobulin antibody is used.

Antimicrobial susceptibility

Antimicrobial susceptibility testing of *T. pallidum* is not available. Penicillin is the drug of choice for treating patients with syphilis. It is the only proven therapy that has been widely used for patients with neurosyphilis, congenital syphilis, and syphilis during pregnancy. Resistant strains have not developed. Long-acting penicillin, such as benzathine penicillin, is preferred. Alternative regimens for patients who are allergic to penicillin and not pregnant include doxycycline and tetracycline. A typical Jarisch-Herxheimer reaction and exacerbation of cutaneous lesions can occur within hours following treatment.

Other treponemal diseases

Three nonvenereal treponemal diseases—yaws, pinta, and endemic syphilis—occur in different geographic locations. These treponematoses are found in developing countries where hygiene is poor, little clothing is worn, and direct skin contact is common because of overcrowding. All three diseases have primary and secondary stages, but tertiary manifestations are not always seen. All diseases respond well to penicillin or tetracycline. These infections are rarely transmitted by sexual contact, and congenital infections do not occur.

Yaws

Yaws is a spirochetel disease caused by *T. pertenue*. It is endemic in the humid, tropical belt, the tropical regions of Africa, parts of South America, India, Indonesia, and many of the Pacific Islands. It is not seen in the United States. The course of yaws resembles that of syphilis, but the early-stage lesions are elevated, granulomatous nodules (hyperkeratotic papilloma).

Endemic syphilis

Endemic syphilis (“bejel”) is caused by *T. pallidum* subsp. *endemicum* and resembles yaws in clinical manifestations. It is characterized by ulcerative lesions on the oropharyngeal mucosa with split papules in the corners of the mouth. It is found in the Middle East and in the arid, hot areas of the world. The primary and secondary lesions are usually papules that often go unnoticed. They can progress to gummas of the skin, bones, and nasopharynx. Dark-field microscopy is not useful because of normal oral spirochetel biota. Poor hygienic conditions are important in perpetuating these infections. Endemic syphilis is transmitted by direct contact or sharing contaminated eating utensils.

Pinta

Pinta, caused by *T. pallidum* subsp. *carateum*, is found in the tropical regions of Central America and South America. It is acquired by person-to-person contact and is rarely transmitted through sexual intercourse. Lesions begin as scaling, painless papules and are followed by an erythematous rash that becomes hypopigmented with time. The tertiary stage presents with hyperchromic, hypochromic, achromic, or dyschromic plaques. It is unique among the pathogenic treponemes in being limited to the skin.

POINTS TO REMEMBER

- Spirochetes are slender, flexuous, helically shaped, motile bacteria.
- Leptospire are most likely to enter the human host through small breaks in the skin or intact mucosa.
- The incubation period of leptospirosis is usually 10 to 12 days but ranges from 3 to 30 days. The onset of clinical illness is generally abrupt, with nonspecific, influenza-like constitutional symptoms, such as fever, chills, headache, severe myalgia, and malaise.
- The pathogenic borreliae are commonly arthropod-borne (by a tick or louse) and cause relapsing fever and Lyme borreliosis (disease).
- *B. recurrentis* and similar species cause relapsing fever. The relapses are caused by immune evasion, including antigenic variation. During a single infection, borreliae systematically change their surface antigens.
- During the febrile period, diagnosis of relapsing fever is readily made by Giemsa or Wright staining of blood smears. Relapsing fever is the only spirochetel disease in which the organisms are visible in blood with bright-field microscopy.
- Laboratory diagnosis of Lyme borreliosis caused by *B. burgdorferi* sensu lato is accomplished by two-tiered serology. Initial reactive or equivocal EIA results are confirmed with an immunoblot or secondary EIA.
- Serologic assays are the most common method for diagnosing syphilis. However, these assays cannot distinguish among the four pathogenic treponemes.
- Among adults, treponemes are transmitted by close, intimate contact. Treponemes can cross the placenta and be transmitted from an infected mother to her fetus. Congenital syphilis affects many body systems and is therefore severe and mutilating. All pregnant women should have serologic testing for syphilis early in pregnancy.

LEARNING ASSESSMENT QUESTIONS

1. What are the general characteristics of spirochetes?
 - a. Slender (0.1 to 0.5 μ m wide)
 - b. Helix-shaped with one or more complete turns in the helix
 - c. Flexible cell wall around which several fibrils are wound
 - d. All of the above
2. Which risk factor is not associated with *Borrelia* spp. endemic relapsing fever?
 - a. Geographic location
 - b. Louse infestation
 - c. Outdoor exposure
 - d. History of tick bites
3. Which tickborne spirochete is associated with a rash or skin lesion?
 - a. *Borrelia burgdorferi* sensu lato
 - b. *Treponema pallidum* subsp. *carateum*
 - c. *Borrelia recurrentis*
 - d. *Leptospira interrogans* sensu lato
4. What is the significance on infectious disease transmission of finding partially engorged ticks attached to skin?
 - a. Pathogen transmission is more probable the longer the vector is attached.
 - b. Pathogen transmission does not occur unless fully engorged.

- c. More than one tick is required for disease transmission.
 - d. There is no significance.
5. What is the typical test of choice for the laboratory diagnosis of relapsing fever?
 - a. Serology
 - b. Dark-field microscopy
 - c. Peripheral blood smear stained with Giemsa stain
 - d. Culture in Ellinghausen-McCullough-Johnson-Harris (EMJH) media
6. A nonreactive result is obtained on a nontreponemal assay performed on serum from a patient suspected of having syphilis. What should be the next step?
 - a. Repeat the nontreponemal assay to confirm the nonreactive result.
 - b. Perform a treponemal assay to confirm the nonreactive result.
 - c. Request a second specimen in 1 week.
 - d. Report the result as nonreactive.
7. Which stage of syphilis is characterized by neurologic symptoms and a reactive treponemal antigen test?
 - a. Primary
 - b. Secondary
 - c. Tertiary
 - d. Latent
8. Where are the most cases of leptospiroses contracted within the United States and its territories?
 - a. Florida
 - b. California
 - c. Virginia
 - d. Puerto Rico
9. Leptospira are typically transmitted from natural hosts, dogs, and rodents to humans via which type of contact?
 - a. Animal bites
 - b. Insect vectors
 - c. Contact with animal feces
 - d. Contact with water contaminated with urine
10. Which of the following are a treponemal antigen serologic test for syphilis? *Select all that apply.*
 - a. VDRL
 - b. FTA
 - c. RPR
 - d. TPPA

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Chlamydia, *Rickettsia*, and similar organisms

Donald C. Lehman

CHAPTER OUTLINE

Chlamydiaceae, 544

General characteristics, 544

Chlamydia trachomatis, 544

Chlamydia pneumoniae, 547

Chlamydia psittaci, 548

Rickettsiaceae and similar organisms, 552

Rickettsia, 552

Orientia, 554

Anaplasmataceae, 555

Coxiella, 556

Bibliography, 557

OBJECTIVES

After reading and studying this chapter, you should be able to:

1. List the members of the family Chlamydiaceae.
2. Discuss the unique growth cycle of *Chlamydia*, describing elementary and reticulate bodies.
3. Describe how the *Chlamydia* and *Rickettsia* differ from other bacteria and viruses.
4. Discuss the most important human diseases caused by the *Chlamydia*, *Rickettsia*, and similar microorganisms.
5. Describe the modes of transmission for each species of *Chlamydia*, *Rickettsia*, and similar microorganisms.
6. Compare the epidemiology and pathogenesis of the serovars of *Chlamydia trachomatis*.
7. Evaluate the available assays for the laboratory diagnosis of *C. trachomatis* and *C. pneumoniae* infections.
8. Discuss the problems with serologic cross-reactivity among the rickettsial species.
9. For the following human rickettsial diseases, compare the causative agents and mode of transmission to humans:
 - Louseborne typhus
 - Rocky Mountain spotted fever
 - Scrub typhus

10. Compare the characteristics and pathogenesis of *Ehrlichia* spp. and *Anaplasma phagocytophilum*.
11. Compare the characteristics of the *Rickettsia* and *Coxiella* and the diseases they cause.
12. Justify the use of serologic assays to diagnose ehrlichiosis and anaplasmosis.

KEY TERMS

Brill-Zinsser disease

Bubo

Elementary body (EB)

Human granulocytic anaplasmosis (HGA)

Human monocytic ehrlichiosis (HME)

Lymphogranuloma venereum (LGV)

Morulae

Pelvic inflammatory disease (PID)

Reiter syndrome

Reticulate body (RB)

Trachoma

Case in point

A 7-day-old female neonate was brought by her grandmother to the emergency department of a large city hospital. She had been discharged 3 days after birth, with the last nursing note indicating that the child was “fussy.” The newborn arrived at the emergency department with a temperature of 39°C, loss of appetite, profuse yellow discharge from the right eye, and general irritability. Medical history revealed the mother to be a 17-year-old intravenous drug abuser with no prenatal care, who had a vaginal delivery in the parking lot of a local hospital. Eye discharge and cell scrapings were cultured. Routine bacterial cultures were negative; however, a rapid nucleic acid amplification test (NAAT) was diagnostic.

Issues to consider

After reading the patient's case history, consider:

- The various organisms that can be recovered from exudative material from newborns
- The clinical infections and disease spectrum associated with these organisms

- How these organisms are transmitted and the risk factors associated with the diseases produced
- The appropriate methods of laboratory diagnosis

This chapter covers obligate intracellular organisms that are either extremely difficult to culture or are nonculturable. Molecular biology assays are used to detect the more commonly seen human pathogens among this group. Their very small size and obligate intracellular parasitism are major characteristics that differentiate the organisms of the genera *Chlamydia*, *Rickettsia*, *Orientia*, *Anaplasma*, and *Ehrlichia* from other bacterial species.

The genus *Chlamydia* is the only genus in the family Chlamydiaceae. A proposal to add the genus *Chlamydophila* was ultimately rejected. The creation of a second genus was somewhat controversial. Therefore readers may find both taxonomic classifications in published literature. Members of the family share characteristics (Table 24.1) and have a unique life cycle. Within the genus *Chlamydia*, four species were previously recognized: *C. pecorum*, *C. pneumoniae*, *C. psittaci*, and *C. trachomatis*. Based on analysis of 16S and 23S ribosomal ribonucleic acid (rRNA) gene sequences, the taxonomic classification was revised. The genus *Chlamydia* consists of the human pathogens *C. trachomatis*, *C. pneumoniae*, and *C. psittaci*. Other named species of *Chlamydia* exist, but they are rarely isolated from humans.

The term *rickettsiae* can specifically refer to the genus *Rickettsia*, or it can refer to a group of organisms included in the order Rickettsiales. There has been significant reorganization in the order Rickettsiales in recent years. The order includes the families Rickettsiaceae and Anaplasmataceae. The family Rickettsiaceae includes the genera *Rickettsia* and *Orientia*. The family Anaplasmataceae includes the genera *Ehrlichia*, *Anaplasma*, *Neorickettsia*, and *Wolbachia*. As a result of this reorganization, *Coxiella* has been removed from the family Rickettsiaceae and placed into the family Coxiellaceae.

Chlamydiaceae

General characteristics

Currently, there are about 15 *Chlamydia* species. As shown in Table 24.2, initial differentiation of the *Chlamydia* spp. associated with human disease was based on selected characteristics of the growth cycle, susceptibility to sulfa drugs, accumulation of glycogen in inclusions, and deoxyribonucleic acid (DNA) relatedness. Table 24.2 also lists additional properties of the Chlamydiaceae species that have helped further differentiate the three human species based on natural host, major diseases, and number of antigenic variants (i.e., serovars).

Chlamydiae are deficient in energy metabolism and are therefore obligate intracellular parasites. Historically, it was believed that the chlamydiae were strictly energy parasites. However, gene sequencing identified the presence of enzymes for the metabolism of glucose 6-phosphate to pyruvate via glycolysis, allowing the bacteria to generate adenosine triphosphate (ATP) via substrate-level phosphorylation. The bacteria depend on the phosphorylated sugar, D-glucose 6-phosphate, from the host cell. Although

chlamydiae do take up ATP from the host cell, they are able to produce their own ATP from D-glucose 6-phosphate also taken from the host cell. The tricarboxylic acid (Krebs) cycle is incomplete in all Chlamydiaceae, and they are unable to synthesize most amino acids, cofactors, and purine and pyrimidine nucleotides.

Their unique growth cycle involves two distinct forms, an **elementary body (EB)**, which is infectious, and a **reticulate body (RB)**, which is noninfectious. The EB has sporelike features in that they are resistant to environmental physical stress. It was believed that EBs are inert; however, recent studies have demonstrated some metabolic activity without cellular division. The growth cycle (Fig. 24.1) begins when the small EB infects the host cell by inducing energy-requiring active phagocytosis where they remain within membrane-bound phagosome. The bacteria prevent interaction of the phagosome with endosomes.

In vivo, host cells are primarily the nonciliated, columnar, or transitional epithelial cells that line the conjunctiva, respiratory tract, urogenital tract, and rectum. During the next 8 hours after cellular penetration, they organize into larger, less dense RBs, which divert the host cell's synthesizing functions to their metabolic needs and begin to multiply by binary fission. About 24 hours after infection, the dividing organisms begin reorganizing into infective EBs. At about 30 hours, multiplication ceases, and by 35 to 40 hours, the disrupted host cell dies, releasing new EBs (Fig. 24.2) that can infect other host cells, continuing the cycle.

The EB has an outer membrane like that of many gram-negative bacteria. The most prominent component of this membrane is the major outer membrane protein (MOMP). The MOMP is a transmembrane protein that contains both species-specific and subspecies-specific epitopes that can be defined by monoclonal antibodies. The *Chlamydia* outer membrane also contains lipopolysaccharide (LPS). This extractable LPS is the primary antigen detectable in genus-specific serologic assays.

Chlamydia trachomatis

C. trachomatis has been divided into two biovars: trachoma and lymphogranuloma venereum. In addition, characterization of the MOMP has separated *C. trachomatis* into 20 serovariants, or serovars (Table 24.3). The trachoma biovar includes serovars A to K. Serovars A, B, Ba, and C are associated with the severe eye infection trachoma, whereas serovars D to K, Da, Ia, and Ja are associated with inclusion conjunctivitis, a milder eye infection, and urogenital infections. Serovars L1, L2, L2a, L2b, and L3 are associated with **lymphogranuloma venereum (LGV)**, an invasive urogenital tract disease. The serovars L are referred to as the biotype LGV, while serovars A to K are called the *trachoma* biovar.

Most *Chlamydia* species carry a plasmid. Analysis of the genus-wide plasmid produced three lineages and indicates that all three evolved from a common ancestor. The plasmids' high level of conservation across the genus implies a strong evolutionary selection for their retention. The plasmid found in *C. trachomatis* is important for virulence and establishing persistent infections, but its role in pathogenesis is unknown. NAATs, such as polymerase chain reaction (PCR), are currently the most commonly used methods to diagnose these infections.

Table 24.1 Comparative properties of microorganisms

Characteristic	Infectious agents				
	Typical bacteria	Chlamydiae	Rickettsiae	Mycoplasmas	Viruses
DNA and RNA	+	+	+	+	–
Obligate intracellular parasites	–	+	+	–	+
Peptidoglycan in cell wall	+	+	–	–	–
Growth on nonliving medium	+	–	–	+	–
Contain ribosomes	+	+	+	+	–
Sensitivity to antimicrobial agents	+	+	+	+	–
Sensitivity to interferon	–	+	–	–	+
Binary fission (replication)	+	+	+	+	–

+, Characteristic is present; –, characteristic is absent.

Table 24.2 Initial differentiation of *Chlamydia* species

Properties	<i>Chlamydia trachomatis</i>	<i>Chlamydia pneumoniae</i>	<i>Chlamydia psittaci</i>
Inclusion morphology	Round, vacuolar	Round, dense	Variable shape, dense
Glycogen in inclusions	+	–	–
Elementary body morphology	Round	Pear-shaped	Round
Sulfa drug sensitivity	+	–	–
DNA relatedness (against <i>C. pneumoniae</i>)	10%	100%	10%
Natural hosts	Humans	Humans	Birds, lower animals
Major human diseases	Sexually transmitted diseases Trachoma Lymphogranuloma venereum	Pneumonia Pharyngitis Bronchitis	Pneumonia FUO

+, Characteristic is present; –, characteristic is absent; FUO, fever of unknown origin.

Clinical infections

Trachoma

C. trachomatis causes the chronic eye infection trachoma (Fig. 24.3). Although cataracts is the leading cause of blindness worldwide, an estimated 1.2 million people currently have blindness caused by trachoma. An estimated 200 million people live in trachoma-endemic areas. In 1996, the World Health Organization began the Alliance for the Global Elimination of Trachoma by the year 2020 (GET2020). Despite widespread success, trachoma is still the number-one cause of preventable blindness in the world.

Trachoma is associated with serotypes A, B, Ba, and C. These serovars are most frequently found near the equator and in climates with high temperature and high humidity; they are not commonly seen in the United States. These serovars produce a chronic infection that, if left untreated, generally results in blindness in adults. Prevention and treatment include antimicrobial therapy, facial cleanliness, environmental improvement, and a simple surgical procedure on the eyelid. Trachoma is a chronic disease that begins as follicular conjunctivitis. The chronic inflammation causes the eyelid to turn inward, which results in continual abrasion to the cornea from the eyelashes. The condition results in scarring and ulceration of the cornea. This can result in secondary bacterial infection and blindness.

Lymphogranuloma venereum

C. trachomatis serovars L1, L2, L2a, L2b, and L3 cause LGV, a sexually transmitted disease (STD); these serovars are more invasive than the others. Patients with LGV present with inguinal and anorectal symptoms (Fig. 24.4). Following an incubation period of 1 to 4 weeks, patients develop a small papule or lesion at the site of infection (e.g., penis, vaginal wall, cervix). This is followed by swelling of the regional lymph nodes. The serovars causing LGV can survive inside mononuclear cells, and the bacteria enter the lymph nodes and produce a strong inflammatory response that often results in fluctuant **bubo** formation and subsequent rupture of the lymph node. Proctitis is common in women because of lymphatic spread of bacteria from the vagina or cervix. Men can develop proctitis as a result of anal-receptive intercourse or lymphatic spread from the urethra. The LGV serovars have also been linked to Parinaud oculoglandular conjunctivitis.

Because LGV is no longer a reportable disease, its prevalence in the United States is unknown; however, it is believed to be uncommon. LGV is usually seen in immigrants and travelers returning from countries in which the disease is endemic, typically the tropics and subtropics. Studies have shown that men who have sex with men and individuals who are infected with the human immunodeficiency virus are disproportionately affected with LGV.

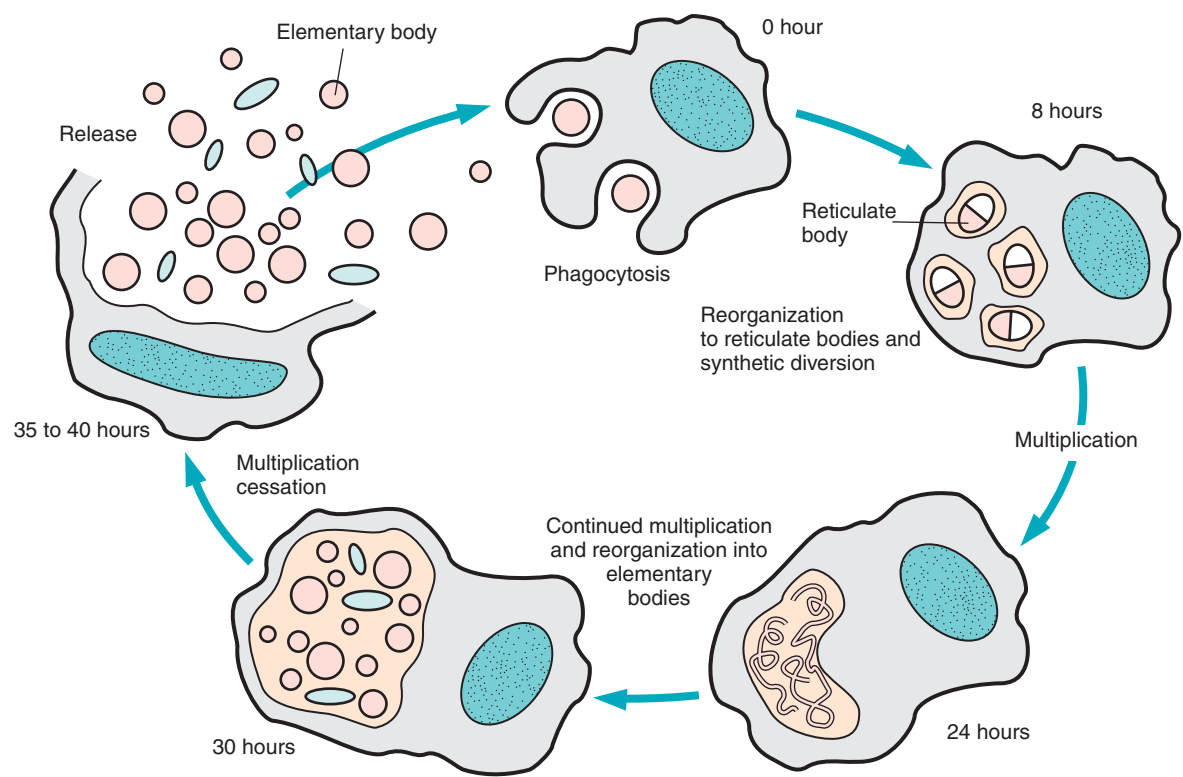


Fig. 24.1 Life cycle of *Chlamydia*.

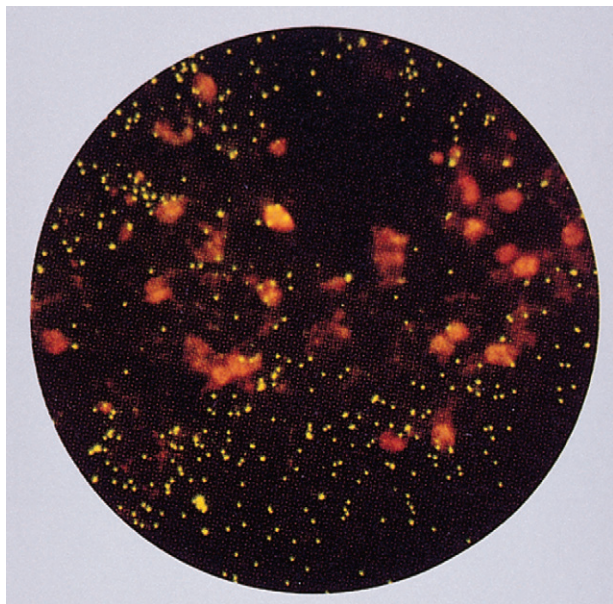


Fig. 24.2 Elementary bodies and cells in a *Chlamydia trachomatis*-positive direct specimen (×400). (Courtesy Syva Microtrak, Palo Alto, CA.)

Other urogenital diseases

C. trachomatis causes urogenital infections in both women and men. Serovars D to K are associated with these clinical infections, which can be persistent and subclinical as well as acute. The same serovars can produce conjunctivitis in both males and females. Typical clinical manifestations of urogenital infection include cervicitis, endometritis, salpingitis, proctitis in women and

Table 24.3 Human diseases caused by <i>Chlamydiaceae</i> species			
Species	Serovars ^a	Disease	Host
<i>Chlamydia trachomatis</i>	A, B, Ba, C	Trachoma	Humans
	D, Da, E, F, G, H, I, Ia, J, Ja, K	Inclusion conjunctivitis (adult and newborn)	Humans
		Nongonococcal urethritis	
		Cervicitis	
		Salpingitis	
		Pelvic inflammatory disease	
		Endometritis	
		Acute urethral syndrome	
		Proctitis	
		Epididymitis	
		Pneumonia of newborns	
		Perihepatitis (Fitz-Hugh–Curtis syndrome)	
	L1, L2, L2a, L2b, L3	Lymphogranuloma venereum	
<i>Chlamydia pneumoniae</i>	1	Pneumonia, bronchitis Pharyngitis Influenza-like febrile illness	Humans
<i>Chlamydia psittaci</i>	10	Psittacosis Endocarditis Abortion	Birds

^aPredominant serovars associated with disease.

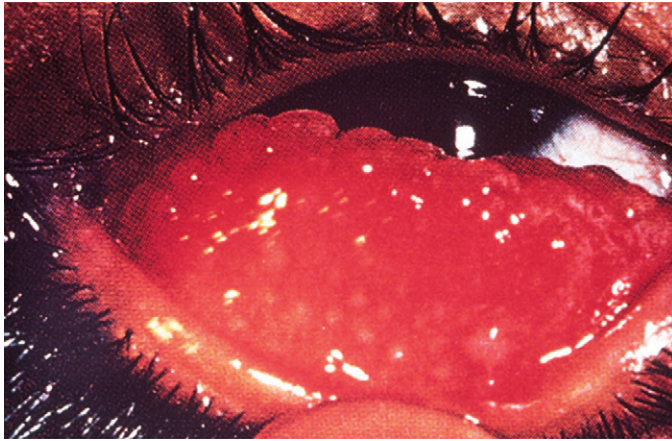


Fig. 24.3 Conjunctival scarring and hyperemic blindness caused by *Chlamydia trachomatis* in ocular infections.



Fig. 24.4 Inguinal swelling and lymphatic drainage caused by *Chlamydia trachomatis* serovars L_1 , L_{2a} , L_{2b} , or L_3 (i.e., lymphogranuloma venereum).

nongonococcal urethritis (NGU), epididymitis, prostatitis, and proctitis in men. **Pelvic inflammatory disease (PID)** and perihepatitis are not uncommon complications in women. Between 45% and 68% of female partners of men with *Chlamydia*-positive NGU yield chlamydial isolates from the cervix. Approximately 50% of current male partners of women with a cervical chlamydial infection are also infected. Most infected women (up to 80%) and some men (approximately 25%) remain asymptomatic, which facilitates spread of bacteria through unprotected sexual contact. Salpingitis can lead to scarring and dysfunction of the oviductal transport system, resulting in infertility or ectopic pregnancy. In the United States, this is a significant cause of sterility. **Reiter syndrome** (urethritis, conjunctivitis, polyarthritides, and mucocutaneous lesions), also known as *reactive arthritis*, is believed to be caused by *C. trachomatis*.

C. trachomatis is the most common sexually transmitted bacterial pathogen and the most common notifiable disease in the United States. In 2020, a total of 1,579,885 cases were reported, a decrease of 13% compared to 2019. This follows an increase of 19.4% from 2014 to 2018. More worrisome is that many infections remain undiagnosed, and the Centers for Disease Control and Prevention (CDC) estimates that 2 million to 3 million new cases occur annually in the United States. It was reported in 2020 that 84% of all reported chlamydia

Table 24.4 Inclusion conjunctivitis in the neonate caused by *Chlamydia trachomatis*

Characteristic	Comments
Incubation period	4–5 days
Signs	Edematous eyelids
Discharge	Copious, yellow
Course	Untreated, weeks to months
Complications	Corneal panus formation, conjunctival scarring

cases in females were among those 15 to 29 years of age. For males, 71% of cases occurred in the same age group. Genital warts, caused by human papillomavirus, is a more common STD in the United States.

Chlamydial infection in the newborn

Because of the high incidence of chlamydia infection in women of childbearing age, health professionals have suggested routine screening of women who are pregnant for colonization. Newborns can be infected with *C. trachomatis* while traveling through an infected birth canal. Chlamydial infection in a newborn delivered by cesarean section is rare, and infection from seronegative mothers has not been reported. Newborns with chlamydial infection can experience conjunctivitis, nasopharyngeal infections, and pneumonia. [Table 24.4](#) shows selected features associated with neonatal inclusion conjunctivitis. The portal of entry is ocular or aspiration, with oropharynx colonization being a necessary event before infection. Infants born in the United States receive prophylactic eye drops, generally erythromycin, to prevent eye infections by *C. trachomatis* and *Neisseria gonorrhoeae*.

Clinically, it is believed that pneumonia in infants younger than 6 months of age is associated with *C. trachomatis*, unless proven otherwise. This pneumonia also can occur as a mixed infection with gonococcus and cytomegalovirus and other viruses. The incubation period for *C. trachomatis* pneumonia is variable, but symptoms generally appear 2 to 3 weeks after birth. Studies have suggested that some cases of sudden infant death syndrome might be caused by *C. trachomatis*.

Case check 24.1

In the Case in Point, the neonate presented with conjunctivitis and symptoms of pneumonia. The signs and symptoms along with the neonate's history are suggestive of *C. trachomatis* infection.

Chlamydia pneumoniae

Chlamydia pneumoniae, formerly known as *Chlamydia* sp. strain TWAR, was originally identified in 1965 from a conjunctival culture of a child (TW) enrolled in a Taiwan trachoma vaccine study. In 1983 at the University of Washington, a similar organism was isolated in HeLa cells from a pharyngeal specimen of a college student (AR). To date, only a single *C. pneumoniae* serovar has been found.

C. pneumoniae is recognized as an important respiratory pathogen thought to be transmitted in human secretions. It is known to be a cause of sinusitis, pharyngitis, acute respiratory

Table 24.5 Summary of key epidemiologic and clinical features of *Chlamydia pneumoniae* infections

Epidemiologic	Clinical
Almost no antibody detectable before 5 years of age	Estimated to account for approximately 6%–10% of outpatient and hospitalized pneumonia; 90% of infections are asymptomatic or mildly symptomatic
Antibodies present in >50% of adults	Biphasic illness—prolonged sore throat, croup-like hoarseness, followed by lower respiratory tract (flulike) symptoms
Attack rate highest between 6 and ≈25 years of age, often focusing on college-age students	Pneumonia and bronchitis, rarely accompanied by sinusitis
No seasonal incidence; epidemics have been reported every 4–6 years	Fever relatively uncommon
Reinfection common	Chest radiograph shows isolated pneumonitis
	One in nine infections results in pneumonia.
	Sarcoidosis, cardiovascular relationships (?)

Table 24.6 Evaluating for *Chlamydia pneumoniae*

Population or situation	Evaluation methods	Comments
Pneumonias requiring hospitalization (age 6–20 years)	<i>C. pneumoniae</i> -specific IgM and IgG: acute and convalescent, use MIF IgM, single visit	12% antibody prevalence
Pharyngitis in college students		9% antibody prevalence
Retrospective, undiagnosed outbreaks in young adults, college students, or military trainees	CF or MIF, IgG specific	
Serious pneumonia, undiagnosed; clinically presents like <i>Mycoplasma pneumoniae</i>	<i>C. pneumoniae</i> -specific IgM and IgG by MIF	If negative, rather than perform cultures for similar respiratory pathogens (i.e., <i>Mycoplasma pneumoniae</i>), reevaluate the patient to determine whether additional testing is necessary

CF, Complement fixation; IgG, immunoglobulin G; IgM, immunoglobulin M; MIF, microimmunofluorescence.

disease, bronchitis, and pneumonia. It also has been isolated from patients with otitis media with effusion, pneumonia with pleural effusion, and aseptic pharyngitis. *C. pneumoniae* can cause a mild atypical pneumonia resembling pneumonia caused by *Mycoplasma pneumoniae*. Probably 90% of infections are asymptomatic or mildly symptomatic, causing a chronic cough and malaise. Hospitalization is rare. Unlike viral respiratory diseases, there seems to be no seasonal incidence. Reinfection with *C. pneumoniae* appears to be common and can be milder or more severe than the initial infection. The epidemiologic and clinical features of *C. pneumoniae* are listed in Table 24.5. The mode of transmission, incubation period, and infectiousness of *C. pneumoniae* infections are still largely unknown. No animal reservoir or vector is known. Table 24.6 summarizes situations and/or populations at risk that would benefit from the detection of *C. pneumoniae*, usually by serologic methods.

The prevalence of *C. pneumoniae* infection is controversial, but infections are thought to be common, with an estimated 200,000 cases per year in the United States. *C. pneumoniae* is regarded as the third most common cause of infectious respiratory disease. It accounts for up to 10% to 15% of community-acquired cases of pneumonia. However, a meta-analysis of 63 research articles published between 1995 and 2019 indicated that *C. pneumoniae* caused a low number of community-acquired pneumonia cases. In PCR studies, the rate of identification of *C. pneumoniae* as a possible pathogen ranged from 0% to 8.95%. Supporting the fact that many infections are asymptomatic or mild, antibodies were demonstrated in 50% to 70% of adults in some populations. It is thought that the attack rate is highest between 6 and 20 years of age, with a particular emphasis in college-age students. Outbreaks have been reported in schools, prisons, and the military.

The clinical picture in college-age students, although it may vary, has a biphasic clinical course. *C. pneumoniae* infection results in prolonged sore throat (5–7 days) and hoarseness, followed by flulike lower respiratory tract symptoms (8–15 days). Because of its striking clinical similarity to bacterial pharyngitis, the result of a streptococcal antigen test often is thought to produce false-negative results. The second phase of the biphasic illness often results in pneumonia (approximately one in nine infections) and bronchitis but is rarely accompanied by sinusitis. Fever is relatively uncommon, and x-rays show isolated pneumonitis.

C. pneumoniae has been implicated as a possible factor in asthma and cardiovascular disease. The organism has been detected in atherosclerotic tissue by culture and NAATs; however, its possible pathogenic role remains under investigation. Association of this organism with other vascular diseases, such as abdominal aortic aneurysm, has also been considered.

Chlamydia psittaci

Chlamydia psittaci is the cause of psittacosis (*psittakos* is the Greek word for parrot) among psittacine birds, also known as *ornithosis* or *parrot fever*. Many avian species can harbor *C. psittaci*. DNA sequencing led to the naming of new species that were formerly considered *C. psittaci*. They include *Chlamydia felis*, *Chlamydia caviae*, and *Chlamydia abortus*.

Inhalation of *C. psittaci* bacteria following contact with poultry is the greatest risk for infection. Person-to-person spread seems unlikely. The bacteria enter the respiratory tract and can spread to the reticuloendothelial cells of the liver and spleen. A lymphocytic inflammatory response occurs in the alveolar and interstitial spaces that can produce edema, necrosis, and bleeding in the lungs. Human infection can

Table 24.7 Appropriate *Chlamydia trachomatis* assays for selected patient populations

Assay	Patient population								Legal applicability (rape or child abuse?)	Test of cure
	Prenatal		Newborn			Clinics ^a				
	Low risk	High risk	Eye	Throat	Plasma	Low risk	High risk			
Culture	A/B	A	B	A	—	A/B	B	Yes	Yes	
Nonculture, nonamplified										
DFA	B	B	A	B	—	B	B	No	No	
EIA	B	B	B	B	—	B	B	No	No	
OIA	—	—	—	—	—	B	B	No	No	
Nonculture, amplified										
PCR, SDA, TMA	IUO	IUO	IUO	IUO	—	A	A	Yes	No	
Serology										
CF	B, LGV	B, LGV	—	—	—	B, LGV	B, LGV	No	No	
EIA	B	B	—	—	B	B	B	NA	No	
MIF	NA	B	—	—	A (IgM)	B	B	No	No	

A, Most useful, stands alone; B, probable, but needs verification or complementary assay recognizing different *Chlamydia trachomatis* macromolecules, i.e., lipopolysaccharide (enzyme immunoassay [EIA]) versus major outer membrane protein (direct fluorescent antibody [DFA]) or competition assay for DNA probes; CFI, complement fixation; IgM, immunoglobulin M; IUO, investigational use only; LGV, lymphogranuloma venereum; MIF, microimmunofluorescence; NA, not available; OIA, optical immunoassay; PCR, polymerase chain reaction; SDA, strand displacement amplification; TMA, transcription-mediated amplification.

^aA low-risk population is defined as one with a less than 5% incidence, such as in an obstetrics-gynecology or family practice patient group (e.g., birth control, annual gynecologic examination). A high-risk population is defined as one with a more than 10% incidence, such as those in sexually transmitted disease clinics, those in university or college student health centers, and emergency department patients.

be asymptomatic or mild, or it can present as a severe, even life-threatening pneumonia. Symptoms include fever, chills, muscle aches, and severe headache. In the United States, fewer than 50 cases of *C. psittaci* are reported annually, although the number of cases might be underreported because infection can be mild. Most cases are associated with occupational exposure by breeding pet birds and working in poultry processing plants, particularly turkey processing plants.

Laboratory diagnosis of chlamydial diseases

There are numerous methods for the laboratory diagnosis of *C. trachomatis* that differ in sensitivity, specificity, negative predictive value (NPV), and positive predictive value (PPV). Table 24.7 identifies the situations in which the tests may be most applicable and identifies the population groups at greatest risk. Table 24.8 provides the predictive values for isolation, detection, and identification methods. The most appropriate tests or combinations of assays used depend on the following factors:

- Knowledge of the population at risk
- Capability and facilities available for testing
- Cost of assays
- Ability to batch specimen types
- Experience of the laboratory scientist

Prevalence in the population to be tested is an important criterion in determining which method or combination of methods should be used. For any assay, the PPV increases (assuming optimal technical conditions) when the prevalence of the disease in the population is high. The type of specimen

selected for laboratory processing depends on the symptoms of the patient and the clinical presentation. Regardless of the source, however, the specimen should consist of infected epithelial cells and not exudate. First morning voided urine for men and vaginal swab specimens for women are excellent for detecting infection. Urine is an acceptable alternative for women.

Dacron, cotton, and calcium alginate swabs can be used, but it should be noted that toxicity has been associated with different lots of each, which is a concern if culture is attempted. Furthermore, it is important to remember that swabs with plastic or metal shafts are superior to those with wooden shafts, which are toxic to cells. Table 24.9 lists the optimal specimens for detection of *Chlamydia* spp. in patients with a variety of clinical manifestations. Specimens for culture must be placed into a transport medium, such as sucrose phosphate glutamate buffer, and transported to the laboratory at 4°C or below within 24 hours. Because molecular biology assays are widely available, cultures are rarely attempted in clinical laboratories.

Specimens collected for the detection of *C. pneumoniae* include nasopharyngeal and throat swabs, sputum, bronchial lavage fluid, and tracheal aspirates (see Table 24.9). *C. psittaci* is most often diagnosed based on the clinical presentation, history of bird exposure, and serologic assays.

Direct microscopic examination

Direct specimen examination by cytologic methods primarily involves trachoma and inclusion conjunctivitis (Fig. 24.5). This method is technically demanding and influenced by the quality of the specimen and expertise of the laboratory scientist. Although this method is difficult to use with

Table 24.8 Detection capabilities of various methods for *Chlamydia trachomatis*

	SENS ^a (%)	SPEC ^a (%)	PPV ^a (%)	NPV ^a (%)	Specimen site				False ±	Reported cross-reactivity	Comments
					Cervical- urethral	Rectal	Urine	Eye			
Culture	50–85	100	73–98	90–100	+	+	+	+	False –	None	Labor-intensive gold standard for specificity
Nonculture, nonamplified											
DFA	75–85	92–98	73–98	95–99	+	+	–	+	False ±	Staphylococci	Screen only; experience in FA needed
EIA	72–95	90–99	45–92	95–99	+	–	LA	LA	False ±	Streptococci, GC, <i>Acinetobacter</i>	Verify with com- plementary assay
Nonculture, amplified											
PCR, SDA, TMA	85–95	99–100	85–95	100	+	–	+		False –	None reported	No verification necessary

^aRange—low to high prevalence as described in the text.

DFA, Direct fluorescent antibody; EIA, enzyme immunoassay; FA, fluorescent antibody; LA, limited availability; NPV, negative predictive value; PCR, polymerase chain reaction; PPV, positive predictive value; SDA, strand displacement amplification; SENS, sensitivity; SPEC, specificity; TMA, transcription-mediated amplification.

Table 24.9 Appropriate specimens for detection of Chlamydial infections

Clinical manifestation, site of infection	Specimen site, type	Comments
Inclusion conjunctivitis and trachoma	Conjunctival swab, scraping with spatula	Specimen collection in neonates is difficult
Urethritis	Urethral swab	In males, insert swab >4 cm; do not use discharge
Epididymitis	Epididymis aspirate	
Cervicitis	Endocervical swab	Remove exudate first
Salpingitis	Fallopian tube (lumen) or biopsy	
Sexually transmitted disease, result clarification	Rectal, vaginal swabs	May be used for supplemental information and in clarifying previous isolates or diagnostic dilemmas
Sexually transmitted disease, male sex partner	Urine	Noninvasive diagnostic procedure; EIA antigen detection is 80% accurate, PCR is 98% accurate
Lymphogranuloma venereum	Bubo or lymph node aspirate	
Infant pneumonia	Throat swab, nasopharyngeal aspirate, or lung tissue	
<i>Chlamydia pneumoniae</i> pneumonia or pharyngitis	Throat washing, throat swab, sputum, bronchial lavage fluid, lung tissue	Tissue culture isolation and direct immunofluorescence are relatively new and need further evaluation
Psittacosis	Sputum, bronchial lavage fluid, pleural fluid, lung tissue	

EIA, Enzyme immunoassay; PCR, polymerase chain reaction.

large numbers of specimens, it does offer rapidity in selected cases, particularly in detecting ocular infection in newborns.

Direct fluorescent antibody (DFA) testing should not be used routinely for genital tract specimens. It is, however, appropriate for rapid diagnosis of inclusion conjunctivitis in infants and sometimes adults. The assay requires an experienced microscopist and is labor-intensive. The sensitivity is 75% to 85%, and cross-reactivity to other bacteria has been reported. The assay

was developed in the 1980s and uses a species-specific epitope on the MOMP. Direct specimen examination offers one additional important advantage—it allows immediate quality control of the specimen, revealing whether columnar epithelial cells are present. Fig. 24.6 shows inclusion bodies demonstrated by direct examination of cytologic stains of endocervical smears. An indirect fluorescent antibody method has been reported for detecting *C. pneumoniae* in respiratory secretions; the antibody

reacts with the MOMP (Fig. 24.7). This same antibody can be used to identify infected cell culture monolayers.

Immunoassays

Direct enzyme immunoassays (EIAs) use monoclonal or polyclonal antibodies directed against the chlamydial LPS. Several commercial kits are available for *C. trachomatis*. They can screen large numbers of specimens, obtain objective results, obtain results in 3 to 5 hours, and use various specimen types. However, none of them equals the sensitivity of culture or NAATs, and many are prone to false-positive results. Because of these limitations, the CDC considers EIAs substandard for the detection of *C. trachomatis*, and they are not recommended for diagnosis.

Cell culture

Until the development of NAATs, chlamydial cell culture was considered the gold standard for detecting *C. trachomatis* infection; however, the usefulness of cell culture has been limited because of the inherent technical complexity, time and specimen handling requirements, expense, biosafety hazard

concerns, and labile nature of the organisms. Even under the most stringent and optimal conditions, isolation of chlamydiae is only approximately 80% sensitive. *C. pneumoniae* can also be recovered in cell cultures. Isolation of *C. psittaci* in culture, although diagnostic, is difficult, dangerous, and not routinely used or recommended.

Fluorescein-labeled monoclonal antibodies can be used to detect the chlamydial inclusions found associated with infected cell lines. Several fluorescent antibodies are available commercially. Some laboratories use species-specific monoclonal antibodies that bind to the MOMP, whereas others prefer the family-specific antibody, which binds to an LPS component. Monoclonal antibodies against the MOMP are reported to offer the brightest fluorescence, with consistent bacterial morphology and less nonspecific staining than monoclonal antibodies against the LPS. Iodine or Giemsa stain can be used, but these methods are less sensitive and specific and are no longer recommended (see Fig. 24.7).

Nucleic acid hybridization and amplification assays

Because of high sensitivity and specificity, the NAATs are the preferred method for the diagnosis of *C. trachomatis* infection. NAATs detect 30% to 50% more *C. trachomatis* infections than culture or earlier nonculture assays. NAATs offer several advantages, including U.S. Food and Drug Administration (FDA) approval to detect *C. trachomatis* in endocervical swabs from women, urethral swabs from men, and urine from men and women. These assays can have the added advantage of detecting two STDs in one sample—gonorrhea and *C. trachomatis* infection. The sensitivity, specificity, NPV, and PPV are higher than those reported for EIAs and cultures. Results can be obtained quickly, and testing is less technically demanding than culture. However, currently no NAAT has been approved for use on conjunctival, oropharyngeal, or rectal specimens. The assays also cannot distinguish LGV strains from other *C. trachomatis* serotypes (A to K).

Five NAATs are currently licensed for use in the United States for the detection of *C. trachomatis* in clinical specimens: the PCR-based Cobas CT/NG (Roche Molecular Systems, Indianapolis, IN); the GenXpert CT/NG (Cepheid, Sunnyvale, CA); the Abbott RealTime m2000 CT/NG (Abbott Molecular, Des Plaines, IL); the APTIMA Combo 2 CT/GC transcription-mediated amplification assay (Hologic, San Diego, CA);

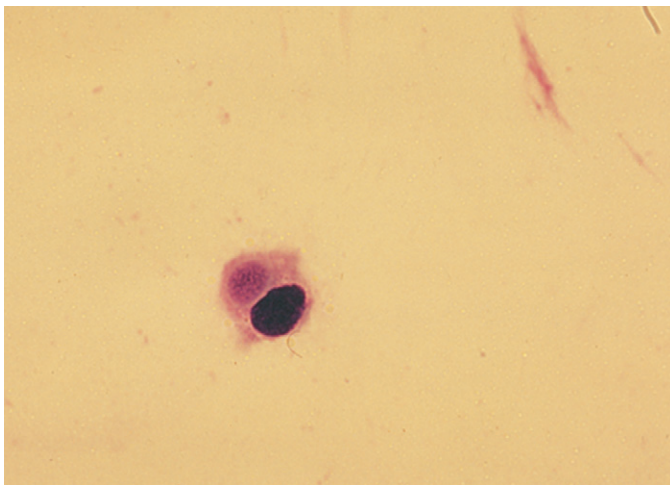


Fig. 24.5 Inclusion body from an ocular swab from a 7-day-old newborn who was discharged but then readmitted with fever, weight loss, lack of eating, and “fussiness.” At 3 days after delivery, *Neisseria gonorrhoeae* was isolated from the ocular discharge, although the patient had been given silver nitrate eye drops. Eye cultures confirmed the presence of *Chlamydia trachomatis* (Giemsa stain, $\times 600$).

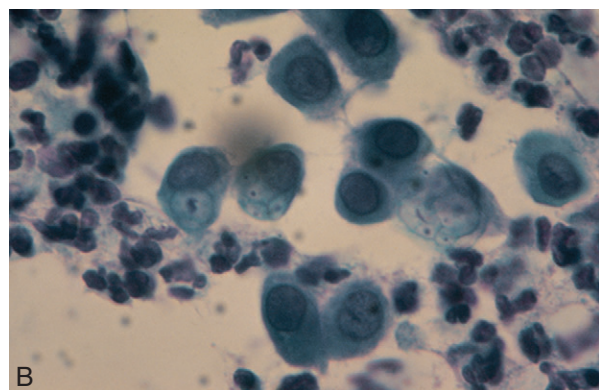
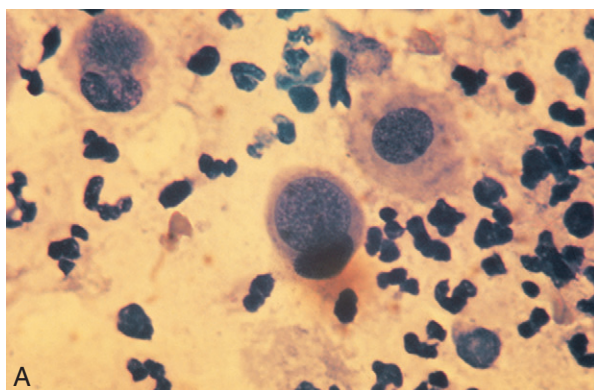


Fig. 24.6 Cytologic examination of endocervical specimens demonstrating inclusion bodies consistent with *Chlamydia trachomatis* (Papanicolaou stain; **A**, $\times 600$; **B**, $\times 600$).

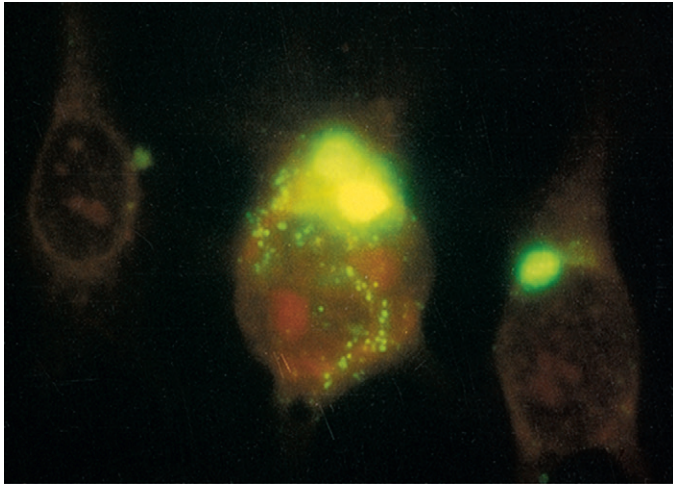


Fig. 24.7 *Chlamydia pneumoniae* detection from a direct sputum smear using a fluorescent-labeled monoclonal antibody, highlighting cytoplasmic inclusion ($\times 400$). (Courtesy DAKO Reagents, Carpinteria, CA.)

and the BD ProbeTec ET strand displacement amplification (BD Diagnostic Systems, Sparks, MD). Although commercial tests differ in their amplification methods and target nucleic acid sequences, the increased sensitivity of NAATs is ascribed to their ability to produce positive signals from as little as a single copy of the target DNA and do not require viable organisms. The assays target sequences on the plasmid, and all five have some degree of automation to facilitate testing and can provide results in the same day. Two NAATs have FDA approval for detecting *C. pneumoniae*; unfortunately, neither method has been thoroughly evaluated for diagnosing infections. Currently, no commercially available NAATs are approved for diagnosing *C. psittaci* infections.

Case check 24.2

In the Case in Point, the diagnosis of *C. trachomatis* infection was confirmed by a NAAT. These assays are generally rapid and highly sensitive and specific. Currently, five FDA-approved commercial NAATs are available; in-house tests can be used if they have been properly validated.

Antibody detection

Serologic assays have little value in the detection of *C. trachomatis* infections. Many individuals have chlamydial antibodies from previous infections, and because chlamydial infections tend to be chronic, it is difficult to demonstrate the traditional fourfold rise in *C. trachomatis* antibody titer between acute and convalescent stage specimens. Serologic testing of uncomplicated genital infections and screening of asymptomatic individuals is not recommended. Infections by the LGV strains are more invasive and therefore more likely to produce a detectable systemic antibody titer. Serologic testing in these patients can support a clinical diagnosis. Detection of high levels of IgM directed against *C. trachomatis* in neonates can be diagnostic of pneumonia.

Also because of high antibody levels in adults, serologic testing has limited value in diagnosing *C. pneumoniae* infections. However, human psittacosis diagnosis is often made by demonstrating a fourfold rise in antibody titer. A single antibody titer greater than 1:32 is suggestive of acute illness

in a symptomatic patient during an outbreak of psittacosis. The increase in the levels of antibodies is usually not demonstrable until the acute illness is over, however, and it is often weak or absent if appropriate antimicrobial therapy is given.

The microimmunofluorescence (MIF) assay has been the most widely used serologic test for the chlamydiae. Demonstration of high IgG titers can help the diagnosis of LGV, epididymitis, ectopic pregnancy, and human psittacosis. Commercial reagents for these assays are available. The MIF assay is considered the method of choice for detecting antibodies to *C. trachomatis*. EIA tests are also commercially available, but they have not been sufficiently evaluated to recommend their routine use.

Rickettsiaceae and similar organisms

The genera *Rickettsia* and *Orientia* are the only two genera in the family Rickettsiaceae. Most members of the rickettsial group are arthropod-borne, obligate intracellular pathogens that can grow only in the cytoplasm of host cells. These bacteria are extremely well adapted to their arthropod hosts. The primary hosts usually have minimal or no disease from their rickettsial infection. The arthropod host allows rickettsiae to persist in nature in two ways. First, rickettsiae are passed through new generations of arthropods by transovarial transmission. Because of this mechanism, arthropods are not only vectors for rickettsioses but also reservoirs. Second, arthropods directly inoculate new hosts with rickettsiae during feeding. An exception to this pattern occurs with *Rickettsia prowazekii*. In this case, the arthropod vector, the body louse, can die of the rickettsial infection, and humans act as a natural reservoir.

Rickettsia

Rickettsiae are short, nonmotile, gram-negative bacilli about 0.8 to $2.0\mu\text{m} \times 0.3$ to $0.5\mu\text{m}$ in size. The members of the genus *Rickettsia* have not been grown in cell-free media but have been grown in the yolk sacs of embryonated eggs and several cell lines. They lack enzymes for sugar metabolism and amino acid, nucleotide, and lipid synthesis. However, they can produce small amounts of ATP via the tricarboxylic acid pathway. Nutritional building blocks (e.g., amino acids) and ATP using an ATP/ADP translocase are acquired from host cells.

At least 30 species of *Rickettsia* are recognized, and most are divided into three groups according to the types of clinical infections they produce and phylogenetic clustering. The typhus group contains only two species: *R. prowazekii* and *R. typhi*. The spotted fever group includes several species generally recognized as human pathogens, such as *R. rickettsii*, *R. parkeri*, *R. philipii*, *R. conorii*, and *R. africae*. The transitional group contains *R. akari*, *R. australis*, and *R. felis*. Because the infective aerosol dose is low, *R. rickettsii*, *R. prowazekii*, *R. typhi*, and *R. conorii* are considered potential bioterrorism agents.

Following the bite of the arthropod vector, the rickettsiae are phagocytosed into endothelial cells (cells that line blood vessels), where they escape from the phagosome and replicate in the cytoplasm of the host cell. Replication in the nucleus also occurs. The rickettsiae pass directly through the plasma membranes of infected cells into adjacent cells without causing damage to the host cells. The rickettsiae are spread hematogenously throughout the host and induce vasculitis in internal organs, including the brain, heart, lungs, and kidneys.

Spotted fever group

Rocky Mountain spotted fever

The most severe of the rickettsial infections, Rocky Mountain spotted fever (RMSF) is caused by *R. rickettsii*. It was first described in the western United States during the latter part of the 19th century. It was not until the early 1900s that researchers demonstrated the infectious nature of the disease, when they infected laboratory animals with the blood of infected patients. The nature of the agent was a mystery because no bacteria were apparent on direct examination or on culture. However, researchers discounted a viral cause because the agent was nonfilterable. The organism was first seen using light microscopy in 1916. Since 2010, the CDC has used the category *spotted fever rickettsiosis* (SFR), which includes RMSF, *Rickettsia parkeri* rickettsiosis, Pacific Coast tick fever (*R. philipii*), and rickettsialpox (*R. akari*). The number of SFR cases in the United States has increased in the past two decades, and between 4500 and 6200 cases of RMSF have been reported annually since 2012.

RMSF is a zoonosis, and humans typically acquire the infection by tick bites. Ticks are the principal vectors and reservoirs for *R. rickettsii*. The most common tick vectors are *Dermacentor variabilis* (Fig. 24.8) in the southeastern United States and *Dermacentor andersoni* in the western part of the country. Other tick species, however, can be vectors. Ticks transmit the organism into humans via saliva, which is passed into the host when the tick feeds. After an incubation period of approximately 7 days, the patient experiences flulike symptoms for approximately 1 week. The symptoms include fever, headache, myalgia, nausea, vomiting, and rash. The rash, which may be hard to distinguish in individuals of color, begins as erythematous patches on the ankles and wrists during the first week of symptoms. The rash can extend to the palms of the hands and soles of the feet but normally does not affect the face. The maculopapular patches eventually consolidate into larger areas of ecchymoses.

Once disseminated, the organisms cause vasculitis in the blood vessels of the lungs, brain, and heart, leading to

pneumonitis, central nervous system (CNS) manifestations, and myocarditis. The patient experiences symptoms secondary to vasculitis, including decreased blood volume, hypotension, and disseminated intravascular coagulation. The mortality rate for untreated or incorrectly treated patients can be as high as 25%, although correct antimicrobial therapy with tetracycline or chloramphenicol lowers the rate to 3% to 6%.

Boutonneuse fever

Boutonneuse fever, also known as *Mediterranean spotted fever*, is caused by *R. conorii* and occurs in France, Spain, and Italy. *R. conorii* also causes Kenya tick typhus, South African tick fever, and Indian tick typhus. Like the agent for RMSF, this rickettsia is tickborne, and its reservoirs include ticks and dogs.

Boutonneuse fever clinically resembles RMSF. The rash involves the palms of the hands and soles of the feet, just as in RMSF. The rash of boutonneuse fever, however, also involves the face. In contrast with RMSF, this disease is characterized by the presence of taches noires (black spots) at the primary site of infection. Taches noires lesions are caused by the introduction of *R. conorii* into the skin of a nonimmune person. As the organism spreads to the blood vessels in the dermis, damage occurs to the endothelium. Edema secondary to increased vascular permeability reduces blood flow to the area and results in local necrosis.

Typhus group

The typhus group of rickettsiae includes the species *R. typhi* (which causes fleaborne typhus, also referred to as *endemic typhus* and *murine typhus*) and *R. prowazekii* (which causes louseborne or *epidemic typhus*). **Brill-Zinsser disease** is a recrudescent disease caused by reactivation of *R. prowazekii* years after epidemic typhus. Generally, the typhus rickettsiae differ from the other rickettsial groups in that they cause cell lysis, thereby releasing the rickettsiae. Other rickettsiae pass directly through an uninjured cell.

Fleaborne typhus

The arthropod vector for *R. typhi* is the oriental rat flea *Xenopsylla cheopis*, and the rat (*Rattus exulans*) is the primary reservoir. The cat flea, *Ctenocephalides felis*, can also harbor the organism. Because this flea infests many domestic and wild animals, it may be an important factor in the persistence of infection in humans. *Rickettsia felis*, described in 1990, also causes fleaborne typhus.

The rickettsiae survive in nature, to a lesser extent, by transovarial transmission. When a flea feeds on an infected host, the rickettsiae enter the flea's midgut, where they replicate in the epithelial cells. They are eventually released into the gut lumen. Humans become infected when fleas defecate on the surface of the skin while feeding. The human host reacts to the bite by scratching the site, allowing direct inoculation of the infected feces into abrasions. *R. typhi* can also be transmitted to humans directly from the flea bite itself.

In the 1940s, approximately 5000 cases of murine typhus were reported annually in the United States. Rigid control measures have reduced that number to fewer than 100 cases annually. The disease essentially occurs only in southern Texas and southern California. However, it continues to be a problem in warmer climates of the world in areas where rats and their fleas are present in urban settings. As is the case



Fig. 24.8 Dorsal view of *Dermacentor variabilis*, the American dog tick, a vector for Rocky Mountain spotted fever ($\times 20,000$). (Courtesy Janice Carr, Centers for Disease Control and Prevention, Atlanta, GA.)

with RMSF, the clinical course of endemic typhus includes fever, headache, and rash. Unlike RMSF, endemic typhus does not always produce a rash; only about 50% of those infected will have a rash. When the rash is present, however, it usually occurs on the trunk and extremities. Rash on the palms of the hands occurs rarely. Complications are rare, and recovery usually occurs without incident. Gastrointestinal symptoms, including anorexia, nausea, vomiting, and abdominal pain, are often present. The fatality rate is about 4% with treatment.

Louseborne typhus

The vectors for louseborne typhus include the human louse (*Pediculus humanus*; Fig. 24.9), squirrel flea (*Orchopeas howardii*), and squirrel louse (*Neohaematopinus sciurioperti*). The reservoirs are primarily humans and flying squirrels located in the eastern United States. The louse often dies because of rickettsemia, unlike vectors of other rickettsiae.

Louseborne typhus is still found commonly in areas of Africa and Central and South America where unsanitary conditions promote the presence of body lice. As seen during World War II, epidemic louseborne typhus can recur even in developed countries when sanitation is disrupted. More than 20,000 cases were documented during the 1980s, with the vast majority originating in Africa. Louseborne typhus resembles the other rickettsioses.

Lice are infected with *R. prowazekii* when feeding on infected humans. The organisms invade the cells lining the gut of the louse. They actively divide and eventually lyse the host cells, spilling the organisms into the gut lumen. When the louse feeds on another human, it defecates, and the infected feces are scratched into skin, just as in murine typhus. Following an incubation period of 1 to 3 days, the disease progression is like that of RMSF, including involvement of the palms of the hands and soles of the feet with the rash. Unlike the case with RMSF, the face may also be affected by rash. Patients have high fever, severe headache, and myalgia. The mortality rates for untreated patients can approach 40%, although mortality rates in treated patients are very low.

Brill-Zinsser disease, also called *recrudescence typhus*, is seen in patients who previously had louseborne typhus. *R. prowazekii* lies dormant in the lymph tissue of the human host until the infection is reactivated. Brill-Zinsser disease is a milder disease compared with louseborne typhus, and death is rare. Patients with latent infections constitute an important reservoir for the organism.



Fig. 24.9 The female head louse, *Pediculus humanus*, which is a vector for *Rickettsia prowazekii*, the agent of epidemic typhus (×40). (Courtesy Dr. Dennis D. Juranek, Centers for Disease Control and Prevention, Atlanta, GA.)

Transitional group

Rickettsialpox

Rickettsialpox is caused by *R. akari*, whose reservoir is the common house mouse, and the vector, the mouse mite *Liponyssoides sanguineus*. Rickettsialpox occurs in Korea and Ukraine and in the eastern United States, including the cities of New York, Boston, and Philadelphia. The infections occur in crowded urban areas where rodents and their mites exist.

Rickettsialpox has similarities to RMSF but is a milder infection and has a biphasic presentation. The rickettsial organism enters the human host following a mite (chigger) bite. The incubation period is about 7 days, after which the first phase presents as a papule at the site of inoculation. The papule progresses to a pustule and then to an indurated eschar. During this state, the bacteria spread hematogenously. This is followed by the second phase, when the patient becomes febrile. The patient also experiences headache, nausea, chills, and a papulovesicular rash. Unlike RMSF, the rash of rickettsialpox appears on the face, trunk, and extremities and does not involve the palms of the hands or soles of the feet. Rickettsialpox symptoms resolve without medical attention in 2 to 3 weeks.

Orientia

Scrub typhus is a disease that occurs in India, Pakistan, Myanmar, eastern Russia, Asia, and Australia. The causative agent is *Orientia* (formerly *Rickettsia*) *tsutsugamushi*. The vector is the *Leptotrombidium deliense* mite, and the reservoir is the rat. Because mites only feed once during their life, they are not considered important reservoirs for human disease. Mites are also reservoirs because the bacteria are transmitted transovarially. The transmission of *O. tsutsugamushi* to the human host is followed by an incubation period of approximately 2 weeks. A tache noire, like that of boutonneuse fever, often forms at the site of inoculation. The normal rickettsial symptoms of fever, severe headache, and rash are also present. The macular to papular rash starts on the trunk and spreads to the extremities but is present on less than 50% of the patients. Unlike the case with RMSF, the rash does not involve the palms of the hands and soles of the feet, and the face is also not involved. Systemic lymphadenopathy, heart failure, and CNS involvement can be present. Without treatment, mortality approaches 30%.

Laboratory diagnosis of rickettsial diseases

Because of their infectious nature, isolation of the rickettsiae is not recommended and should be attempted only by biosafety level 3 laboratories. If culture is attempted, blood should be collected as early in the disease course as possible. Methods for immunohistochemical testing of tissue specimens have been established for several rickettsial diseases. Monoclonal antibodies directed against the spotted fever or typhus group have been used, but no antibody is commercially available. NAATs are particularly useful for eschar specimens. Only NAATs and immunohistochemical assays can provide a diagnosis in the acute stage, when treatment is critical. Unfortunately, neither method is readily available.

Typically, serologic assays are the only laboratory tests performed for the diagnosis of rickettsial diseases. Unfortunately, these methods can confirm a diagnosis only

in convalescent specimens and offer little help in diagnosing acute infections that could guide antimicrobial therapy. An acute blood sample should be taken as early as possible, and a second sample should be taken 1 to 2 weeks later. If the second sample does not detect a fourfold or greater increase in titer, a third sample is recommended 4 weeks after onset of symptoms.

The immunofluorescent antibody (IFA) test is considered the gold standard method for antibody detection. Because of cross-reactivity among members of the same groups (spotted fever and typhus), generally only group-specific antibody testing is available. Antibodies to certain rickettsial species are known to cross-react with bacteria in the genus *Proteus*. This gave rise to the Weil-Felix agglutination test. Because this assay does not use rickettsial antigens, it is nonspecific and rarely used in the United States. However, because of its low cost, it is used in some other countries. An agglutination test using latex beads coated with rickettsial antigens is commercially available for the diagnosis of RMSF (Panbio, Baltimore, MD) as are EIAs.

Anaplasmataceae

The family Anaplasmataceae contains several genera of importance to human and veterinary medicine. *Anaplasma* and *Ehrlichia* are the most significant for human disease. Members of the family are small, nonmotile, obligate intracellular bacteria. They replicate within membrane-derived vacuoles within the cytoplasm. Most members of the family grow in vertebrate hosts and one invertebrate vector. In vertebrate hosts, they reside in cells of hematopoietic origin.

Ehrlichia

Ehrlichiosis was first noted in France in the 1930s in dogs. Postmortem examination revealed rickettsial-like inclusions in the monocytes of the dead animals. These rickettsiae were named *Rickettsia canis*. They were obligately intracellular, arthropod-borne coccobacilli. They differ from members of the rickettsiae in that they multiply in the phagosomes of host leukocytes and in other cells derived from the bone marrow, not in the cytoplasm of endothelial cells.

Because these organisms grew within host cell vacuoles, they were reclassified into a new genus, *Ehrlichia*, in 1945. The ehrlichiae have a developmental cycle like that of the chlamydiae. There are two forms: the denser, infective EB, and the RB that replicates in the phagosome and prevents phagolysosome formation. As the cells divide within the phagosome, they develop into **morulae** (Latin for “mulberry”; Fig. 24.10). Morulae are round to oval clusters of bacteria 1 to 3 μm in diameter that stain blue in Wright’s and Giemsa stains. As the host cell ruptures, the morulae break into many individual EBs, which continue the infective cycle.

Ehrlichia chaffeensis causes *E. chaffeensis* ehrlichiosis, formerly called **human monocytic ehrlichiosis** because the bacteria primarily reside in mononuclear cells such as monocytes. Most cases occur in the United States, but some cases are reported in Europe, Africa, and South and Central America. In the United States, about 1600 cases are reported annually, and most cases are found in the southeastern and south-central states as well as in the Mid-Atlantic states. In 2019, four states, Missouri, Arkansas, New York, and North Carolina, accounted for over one half of all reported cases of

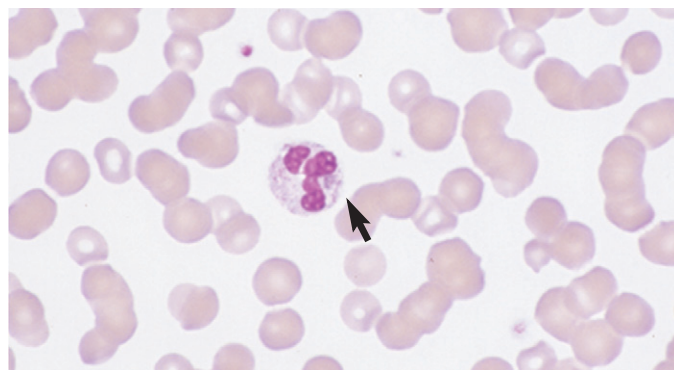


Fig. 24.10 *Anaplasma* morula (arrow) in an infected white blood cell ($\times 1000$).

ehrlichiosis. Most cases occur in the summer months June and July, which is expected for a tickborne disease. *E. chaffeensis* causes the most cases, but *Ehrlichia ewingii*, the second most common, produces a disease indistinguishable from *E. chaffeensis*. Currently, no serologic test can distinguish these agents.

The seroprevalence of *E. chaffeensis* ranges from 1.3% to 12.5% in Arkansas and Tennessee, two states with a high incidence of infection. This indicates that cases may be underreported. Natural hosts of the organism include dogs, deer, and humans, with the lone star tick (*Amblyomma americanum*) being the primary vector. This tick also transmits *E. ewingii*. *E. muris eauclairensis* is the third most common cause of ehrlichiosis in the United States, with most cases occurring in Minnesota and Wisconsin, and is transmitted by the black-legged tick (*Ixodes scapularis*).

Many patients with *E. chaffeensis* ehrlichiosis experience asymptomatic infection. The organism has an incubation period of 5 to 10 days. Patients often experience fever, headache, malaise, myalgia, and nausea. Vomiting, diarrhea, cough, and joint pain are occasionally present. A petechial, macular, or maculopapular rash is sometimes present (57%) in pediatric patients infected with *E. chaffeensis*; however, adults rarely experience a rash (<30%). Patients may also have evidence of leukopenia and neutropenia, thrombocytopenia, and elevated liver enzyme levels. Patients can experience severe complications, including a toxic shock-like syndrome presentation, CNS involvement, and acute respiratory distress syndrome. Mortality rates are approximately 2% to 3%.

Direct staining (Giemsa or Wright) of peripheral blood smears or buffy coats for morulae can be used for diagnosing *E. chaffeensis* infections; however, this method is not very sensitive (29%). The bacteria are primarily found in monocytes, and the percentage of cells infected is less than 10%. Antigen detection in tissues, such as bone marrow, liver, and spleen, has been described. Again, the sensitivity is low (40%), and cross-reaction with other species has been noted. This leaves NAATs as a frequently used method for direct detection of *E. chaffeensis* from whole blood. The use of real-time PCR and multiplex PCR has been described. The bacteria can be isolated from peripheral blood in cell culture, but this method is rarely used. Most cases of *E. chaffeensis* ehrlichiosis are diagnosed retrospectively by serologic testing demonstrating a fourfold increase in IgG antibody. The IFA test is the most widely used method. Significant antibody cross-reactivity has been seen among *Ehrlichia* spp. and between *Ehrlichia* and *Anaplasma*.

Anaplasma

Anaplasma phagocytophilum, formerly known as *Ehrlichia phagocytophilum*, causes the disease **human granulocytic anaplasmosis (HGA)**. The incubation period for HGA is 1 to 5 days. The symptoms closely resemble those of *E. chaffeensis* ehrlichiosis and include fever, headache, malaise, and myalgia. Less than 10% of infected individuals have a rash.

The number of HGA cases is probably underreported. Annually, about 4000 cases are reported in the United States, with most cases occurring in the upper Northeast region. In 2018, the highest rates per 100,000 people were in Vermont (391), Maine (355), New Hampshire (158), and Rhode Island (141). Significant infection rates also occur in Minnesota (88) and Wisconsin (63). Natural hosts include deer, rodents, horses, cattle, and humans. Tick vectors include *I. scapularis* in the northeast and *Ixodes pacificus* along the west coast. The geographic range of anaplasmosis is increasing corresponding to the blacklegged tick's expanding range.

Typical laboratory test results include elevated transaminase levels, thrombocytopenia, and leukopenia with lymphopenia. As with *E. chaffeensis* ehrlichiosis, staining of peripheral blood buffy coats can be used to diagnose HGA. The morulae are found in granulocytes (see Fig. 24.10), and the sensitivity is about 60%. The percentage of granulocytes infected can reach 40%. Diagnosis can also be made by using direct antigen detection, NAATs, and isolation in cell cultures. IFA serologic kits are available for the detection of antibodies to *A. phagocytophilum*.

Coxiella

Coxiella burnetii is the only species in the genus. This organism differs in several ways from many members of the families Rickettsiaceae and Anaplasmataceae. For example, *C. burnetii* has been grown in cell-free media and should be considered a facultative intracellular parasite. The bacterium does not transport ATP across its plasma membrane, and it develops within the phagolysosomes of infected cells. The acidic environment activates its metabolic enzymes. The bacteria exist in two forms. The large-cell variant is rod shaped (0.4–1.5 µm) and metabolically active inside host cells. Under adverse conditions, the large-cell variant converts into the denser small-cell variant (0.22 µm) that survives harsh environmental conditions. Another distinguishing feature is that *C. burnetii* is generally not transmitted by arthropods, although it is known to infect more than 12 genera of ticks and other arthropods. The bacteria can infect fishes, birds, rodents, livestock, and other mammals.

C. burnetii is the causative agent of Q (query) fever, a disease found worldwide. Roughly 160 cases are reported annually in the United States. Q fever is highly contagious and, as such, is considered a potential bioterrorism agent (see Chapter 30). Most infections are spread by the inhalation of dried birthing fluids of several animals. Therefore occupational hazards include ranching or livestock management. The ingestion of unpasteurized milk is also a recognized risk factor. Approximately 50% of infected individuals will remain asymptomatic. In the remaining cases after an incubation period of 2 to 3 weeks, acute Q fever generally has an abrupt onset of an undifferentiated febrile disease consisting

of high fever that can be accompanied by chills, headaches, myalgia, arthralgia, cough, and rarely, a rash. Patients may present with elevated liver enzyme levels, increased erythrocytic sedimentation rate, and thrombocytopenia. Because of the rapid dissemination of the bacteria, several tissues can be infected. Chronic disease occurs in a small number of patients and can result in vascular disease, endocarditis, and bone and joint infections.

Because the symptoms vary among patients and can be difficult to distinguish from other diseases, it can be a challenging disease to diagnose. The laboratory diagnosis of Q fever can be made by direct IFA of infected tissue. However, except for heart tissue in cases of endocarditis, infected tissue contains low numbers of bacteria. NAATs, such as the PCR assay, have also been successful in diagnosing acute and chronic infections; whole blood and buffy coats are often successful in detecting the organism. However, NAAT sensitivity drops significantly after antibody production, which occurs about 2 weeks after infection. *C. burnetii* is highly contagious; isolation in cell cultures should be attempted only in biosafety level 3 facilities. Several serologic assays have been described for detecting antibodies in acute and chronic cases. The IFA test is the method of choice. EIA kits are commercially available and FDA approved and have sensitivities and specificities comparable with those of the IFA test.

POINTS TO REMEMBER

- Chlamydiae and rickettsiae are obligate intracellular organisms.
- *Chlamydia trachomatis* is the most common sexually transmitted bacterial pathogen, and certain serovars are associated with trachoma, which can result in blindness.
- The LGV strains of *C. trachomatis* are more invasive, producing a more serious infection and pronounced antibody response.
- NAATs are better assays for the diagnosis of *C. trachomatis* infections compared with cultures.
- *C. pneumoniae* is a relatively common respiratory tract pathogen considered responsible for many cases of community-acquired pneumonia.
- *C. psittaci* is the cause of psittacosis, also known as *parrot fever* or *ornithosis*. This bacterium produces lower respiratory tract infections in humans.
- The *Rickettsia* spp. are important human pathogens responsible for several diseases, including RMSF, rickettsialpox, and typhus.
- The *Rickettsia*, *Orientia*, *Ehrlichia*, and *Anaplasma* are typically transmitted to humans by arthropod bites.
- *Ehrlichia* and *Anaplasma* are intracellular parasites of white blood cells—mononuclear cells and granulocytes, respectively.
- *Coxiella burnetii* is the causative agent of Q fever. Infection is most often transmitted by inhalation of dried birthing fluids. The ingestion of unpasteurized milk is also a risk factor.
- *C. burnetii* differs from the rickettsiae by developing within the phagolysosome of infected cells and is generally not transmitted by arthropods. *C. burnetii*, unlike the *Rickettsia* spp., has been grown in cell-free media.

LEARNING ASSESSMENT QUESTIONS

- Which organisms should be considered as probable causes of neonatal conjunctivitis?
 - Chlamydia trachomatis* and *Chlamydia pneumoniae*
 - Chlamydia pneumoniae* and *Chlamydia psittaci*
 - Chlamydia trachomatis* and *Neisseria gonorrhoeae*
 - Chlamydia pneumoniae* and *Neisseria gonorrhoeae*
- Which *Chlamydia trachomatis* serotypes are associated with lymphogranuloma venereum?
 - A, B, and C
 - A to K
 - D to K
 - L1, L2, and L3
- How does psittacosis or ornithosis present in human infections?
 - Endocarditis
 - Pneumonia
 - Nausea and vomiting
 - Tissue abscess
- What is the most common laboratory method used to diagnose rickettsial diseases?
 - Serology
 - Growth in cell monolayers
 - Direct immunofluorescent assay
 - Histologic tissue stain with Giemsa stain
- Which cells do the *Ehrlichia* typically infect in humans?
 - Hematopoietic stem cells
 - Reticulocytes
 - Granulocytes
 - Monocytes
- During human infection by the rickettsiae, where do the bacteria usually replicate?
 - Attached to the outside of the plasma membrane
 - Within the endosome
 - Free in cytoplasm
 - Within a phagolysosome
- Which stain should be performed on a conjunctival scraping for microscopic examination for the diagnosis of inclusion conjunctivitis?
 - Gram
 - Giemsa
 - Machibavello
 - Fluorescent antibody
- Which time of year do most cases of Rocky Mountain spotted fever occur in the United States?
 - Winter
 - Spring
 - Summer
 - Fall
- Areas of overcrowding and poor personal hygiene are known for a high incidence of infections by which organism?
 - Rickettsia prowazekii*
 - Rickettsia coronii*
 - Rickettsia akari*
 - Rickettsia typhi*
- In July, a 28-year-old male patient presents to his primary care provider complaining of headache, malaise, and myalgia. He is also found to have a fever, but no rash is present. His medical history is unremarkable, and he reports that he recently returned from a camping trip in Maine. A complete blood count revealed leukopenia, and clumps of

blue inclusions about 2 μ m in diameter were found in the neutrophils. What is the most likely diagnosis?

- Ehrlichia chaffeensis* ehrlichiosis
 - Human granulocytic anaplasmosis
 - Flea-borne typhus
 - Q fever
- For the neonate described in the Case in Point, what other clinical conditions could have resulted from infection with the causative organisms?
 - How does lymphogranuloma venereum differ from other sexually transmitted diseases caused by *Chlamydia trachomatis*?
 - Which types of infections are associated with *Chlamydia pneumoniae*?
 - How does *Coxiella burnetii* differ from the *Rickettsia* spp.?

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Mycoplasma and Ureaplasma

Donald C. Lehman and Connie R. Mahon

CHAPTER OUTLINE

General characteristics, 559

Clinical infections, 560

Mycoplasma pneumoniae, 560

Genitourinary tract infections associated with the mollicutes, 561

Mycoplasma fermentans, 562

Laboratory diagnosis, 562

Specimen collection and transport, 562

Nucleic acid amplification tests, 563

Culture, 563

Serologic diagnosis, 566

Antimicrobial susceptibility, 567

Interpretation of laboratory results, 567

Bibliography, 569

9. Justify the use of serologic assays for diagnosing *M. pneumoniae* infections.
10. Describe two selective media for the detection of the mycoplasmas.
11. Evaluate the use of different classes of antimicrobial agents to treat mycoplasmal infections.
12. Provide recommendations for the proper interpretation and reporting for *Mycoplasma* and *Ureaplasma*.

KEY TERMS

Cell wall deficient

L-forms

Pleuropneumonia-like organism (PPLO)

Nongonococcal urethritis (NGU)

Pelvic inflammatory disease (PID)

Primary atypical pneumonia

T-strain mycoplasma

OBJECTIVES

After reading and studying this chapter, you should be able to:

1. Describe the general characteristics of the *Mycoplasma* and *Ureaplasma*, and how they differ from other bacterial species.
2. Name the clinical specimens from which the mycoplasma species are most likely to be isolated.
3. Compare the clinical diseases caused by *Mycoplasma pneumoniae*, *Mycoplasma hominis*, *Mycoplasma genitalium*, *Mycoplasma fermentans*, and *Ureaplasma urealyticum*.
4. Evaluate the recovery of *M. hominis* from a genital specimen.
5. Compare the pneumonia caused by *Mycoplasma pneumoniae* with that caused by *Streptococcus pneumoniae*.
6. Discuss the possible roles of *M. hominis* and *U. urealyticum* in infections of low-birth-weight and high-risk neonates.
7. Discuss the clinical manifestations of *Mycoplasma* spp. in immunocompromised patients.
8. Analyze the diagnostic methods appropriate for the detection and identification of mycoplasmal and ureaplasma infections including specimen collection, transport, storage, and appropriate media.

Case in point

A premature male neonate in the neonatal intensive care unit, who weighed 1.5 lb at birth (low birth weight), developed signs of meningitis, and a lumbar puncture was performed. The results of a white blood cell count of cerebrospinal fluid (CSF) were negative. The Gram-stained smear showed "no organisms seen," and the result of routine culture at 3 days was reported as "no growth." The infant was still symptomatic at this time, and the pediatric infectious disease physician, after consultation with the clinical microbiologist, performed another lumbar puncture and ordered additional cultures. An organism was recovered by the laboratory.

Issues to consider

After reading the patient's case history, consider:

- The cause of meningeal infections in the given patient population
- Supporting laboratory findings and how they help establish the diagnosis
- Methods for recovery of the suspected causative agent

This chapter discusses bacteria within the class Mollicutes, the smallest self-replicating organisms once thought to be viruses because their small size allowed them to pass through filters, hence the name *filterable*. Members of this class are also referred to by the common names *mollicute* or *mycoplasma*. This group of bacteria is characterized by permanently lacking a cell wall. They range in size for coccoid forms from approximately 0.2 to 0.3 μm in diameter to tapered rods of approximately 1 to 2 μm in length and 0.2 to 0.3 μm in diameter. Seven families, at least 12 genera, and over 200 species of mollicutes have been described. At least 16 species of mollicutes have been isolated from humans.

Mycoplasma and *Ureaplasma* are two of the four genera in the family Mycoplasmataceae. Numerous species of *Mycoplasma* and *Ureaplasma* have been identified in plants and animals; however, the following species are the most significant human pathogens (Table 25.1):

- *Mycoplasma pneumoniae*, which causes respiratory disease
- *Mycoplasma hominis*, associated with urogenital tract disease
- *Ureaplasma urealyticum*, associated with urogenital tract disease

The family Acholeplasmataceae contains five genera, including *Acholeplasma*. Members of this genus do not appear to cause significant infections in humans.

General characteristics

The first *Mycoplasma* sp. was isolated in the late 1800s from a cow with pleuropneumonia. Later, a mycoplasma was isolated from humans and was referred to as a **pleuropneumonia-like organism (PPLO)** and the *Eaton agent*, after the researcher who first isolated it from humans. This human isolate was ultimately named *Mycoplasma pneumoniae*. Because of the absence of a cell wall, the mycoplasmas are pleomorphic and are not visible microscopically with the Gram stain. In addition, this characteristic makes them resistant to cell wall-active antibiotics, such as the penicillins and cephalosporins. They were originally grouped under the general term **cell wall-deficient** bacteria given the absence of a cell wall. They are not, however, classified as **L-forms**, which are bacteria that have temporarily lost their cell wall attributable to environmental conditions. Table 25.2 compares features of the three genera found in human specimens.

Mycoplasmas are generally slow-growing, highly fastidious, facultative anaerobes requiring complex media containing nucleic acid precursors, cholesterol, and fatty acids for growth; important exceptions include aerobic *M. pneumoniae* and the more rapidly growing *M. hominis*. The *Acholeplasma* do not require cholesterol supplements in laboratory media. The mycoplasmas produce small colonies ranging in size from about 15 μm to over 300 μm in diameter. *Mycoplasma* spp. often grow embedded beneath the surface of solid media; therefore transferring colonies with a loop is ineffective. On solid media, some species (e.g., *M. hominis*) form colonies with slightly raised centers, giving the classic “fried egg” appearance (Fig. 25.1). In the laboratory, mycoplasmas are common and hard to detect contaminants of cell cultures.

Table 25.2 Human clinical isolates in the class Mollicutes

Feature	<i>Mycoplasma</i>	<i>Ureaplasma</i>	<i>Acholeplasma</i>
Cell wall deficient	+	+	+
Gram stain	–	–	–
Penicillin susceptible	–	–	–
Urease activity	–	+	–
Lack of cell wall induced in hypertonic solution and by penicillin, lysozyme, or salts	–	–	–
Exists in nature as a free-living organism	–	–	+
Pleomorphic shape	+	+	–
Other shared characteristics	Smaller than other bacteria; close in size to myxoviruses		
	Smaller genome than other bacteria		
	Lower guanidine-to-cytosine ratio than most bacteria		
	Limited metabolic activity (i.e., fastidious)		
	Many mollicutes contain DNase		
+, Feature present; –, feature absent.			

Table 25.1 Divergent ecosystems inhabited by genera of the class Mollicutes

Ecosystem	<i>Mycoplasma</i>	<i>Ureaplasma</i>	<i>Acholeplasma</i>	<i>Spiroplasma</i>	<i>Anaeroplasm</i>
Soil and grasses	–	–	–	–	–
Crops and plants	–	–	–	+	–
Mown hay	–	–	–	–	–
Water	–	–	+	–	–
Deciduous trees	–	–	–	+	–
Humans	+	+	–	–	+
Cattle	+	+	–	–	+
+, Present in ecosystem; –, rarely associated with ecosystem.					

The mycoplasmas adhere to the epithelium of mucosal surfaces in the respiratory and urogenital tracts and are not eliminated by mucous secretions or urine flow. Fig. 25.2 depicts electron micrographs of ciliated tracheal epithelial cells before and after *M. pneumoniae* adherence. *M. pneumoniae* utilizes the P1 adhesion and other proteins to adhere to host cells of the respiratory tract. P1 is associated with a polar extension called an attachment organelle or foot. The organelle is responsible for cytoadherence. This ability of *M. pneumoniae* to adhere and attach to hosts' cells is crucial for the bacteria to survive and infect. Once attached, the host's mucociliary clearance mechanisms are compromised and prevented from eliminating the organism, damaging the respiratory epithelial cells. Fig. 25.3 is an electron micrograph demonstrating the shape of *M. pneumoniae* and its orientation of attachment. Although *M. pneumoniae* mainly inhabits the surface of the respiratory epithelial cells, it may also invade tissues and replicate intracellularly.

Mycoplasma spp. indigenous to humans are listed in Table 25.3. The human mollicutes are susceptible to adverse environmental conditions, such as heat and drying. Transmission of mollicutes in humans can occur via direct sexual contact,

from mother to child during delivery or in utero, and by respiratory secretions or fomites in cases of *M. pneumoniae* infections.

Clinical infections

Mycoplasma pneumoniae

M. pneumoniae does not occur as a normal commensal organism; therefore its isolation is always significant and pathognomonic. Infection can result in a relatively common pneumonia known as **primary atypical pneumonia** or *walking pneumonia*. The clinical manifestations resemble those caused by *Chlamydia pneumoniae*. The disease differs from the typical pneumonia caused by *Streptococcus pneumoniae* in that it is milder and has a higher incidence in young adults. Although infection is not considered seasonal, more cases occur in autumn and early winter. Outbreaks also have been noted when adolescents return to school in the fall.

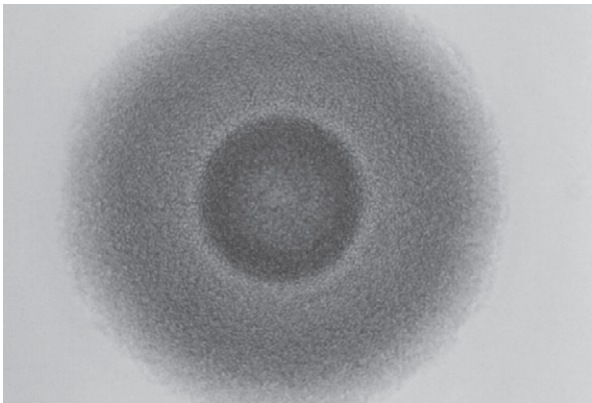


Fig. 25.1 Typical large *Mycoplasma* colony showing "fried egg" appearance. (Courtesy Bionique Testing Laboratories, Saranac Lake, NY)

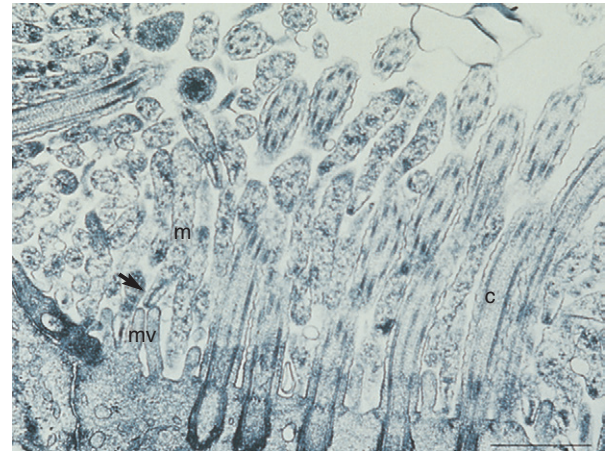


Fig. 25.3 Electron micrograph of *Mycoplasma pneumoniae* attaching by specific attachment features to ciliated trachea ($\times 100,000$). Arrow indicates attachment organelle. c, Cilia; m, mycoplasma; mv, microvilli.



Fig. 25.2 Electron micrographs showing the effect of *Mycoplasma pneumoniae* on ciliated tracheal cells. **A**, Infected animal model. **B**, Uninfected animal model ($\times 20,000$).

Table 25.3 *Mycoplasma* species indigenous to humans

Mycoplasma species	Usual habitat	Reported frequency	Colony morphology ^a
<i>M. salivarium</i>	Oropharynx	Very common	Large, "fried egg"
<i>M. orale</i>	Oropharynx	Common	Small, spherical
<i>M. buccale</i>	Oropharynx	Uncommon	Large, fried egg
<i>M. faucium</i>	Oropharynx	Uncommon	Large, fried egg
<i>M. lipophilum</i>	Oropharynx	Rare	Large, fried egg
<i>M. pneumoniae</i>	Oropharynx	Common (with disease)	Small, spherical, granular
<i>M. fermentans</i>	Oropharynx	Rare	Small, fried egg, spherical
<i>M. hominis</i>	Urogenital tract	Uncommon	Large, fried egg, vesiculated peripheral zone
	Oropharynx	Uncommon	
<i>M. genitalium</i>	Urogenital tract	Common (9%–50% of women)	Tiny, spherical, fried egg, granular
	Urogenital tract	Occasional	
<i>Ureaplasma urealyticum</i>	Urogenital tract	Very common (81% of women; 30%–50% of men)	Tiny, spherical, fried egg, granular
<i>Acholeplasma laidlawii</i>	Oropharynx	Rare	Small, fried egg, spherical

^aRelative sizes: large, greater than 100 nm; small, 50 to 100 nm; tiny, less than 50 nm.

M. pneumoniae is extremely susceptible to desiccation because it lacks a cell wall; hence, transmission is probably through aerosol droplet spray produced while coughing.

M. pneumoniae causes 4% to 8% of reported pneumonias in the general population and up to 70% in confined populations, such as those in military settings, prisoners, and college students. School-age children and young adults are especially susceptible to infection. Epidemics are known to occur in these populations. Historically, clinical disease was uncommon in very young children and older adults. However, recent outbreaks and endemicity have been reported in older adults and children younger than 5 years of age. A recent study found that 40% of 830 Chinese children with community-acquired pneumonia tested positive for *M. pneumoniae* by polymerase chain reaction (PCR).

Many infections are asymptomatic or very mild. The most common presentation is tracheobronchitis often accompanied by pharyngitis. About one third of infected patients demonstrate clinically apparent pneumonia (Fig. 25.4). The incubation period is usually 2 to 3 weeks, and early symptoms are nonspecific, consisting of headache, low-grade fever, malaise, and anorexia. Dry cough and earache are accompanying symptoms. Extrapulmonary complications, including cardiovascular, central nervous system, dermatologic, and gastrointestinal problems, are rare occurrences. In addition, *Mycoplasma pneumoniae* infection has been associated with chronic lung disease and bronchial asthma. *M. pneumoniae* is not associated with infections of the urogenital tract. It has, however, been implicated as a co-infection or cofactor in epidemic group A meningococcal meningitis (*Neisseria meningitidis*) and infant pneumonitis. Recently, a unique virulence factor known as community acquired respiratory distress syndrome (CARDS) toxin has been identified and associated in the pathogenesis of *M. pneumoniae* infections. It appears that the CARDS toxin helps in the colonization and development of disease, causing an inflammatory response and subsequent airway dysfunction.

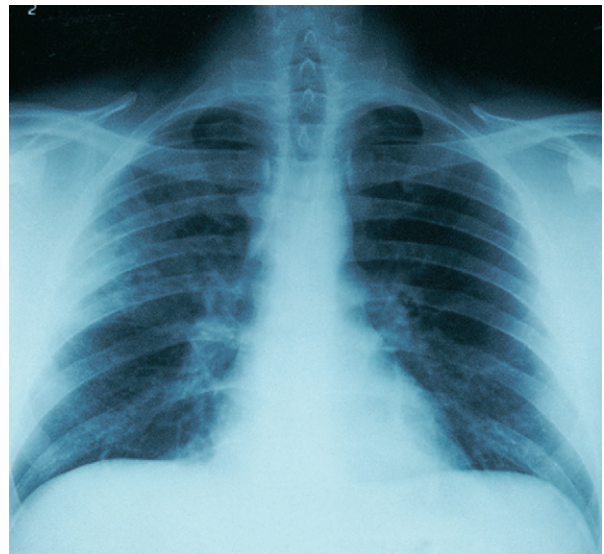


Fig. 25.4 Typical chest x-ray of a patient with a 3-week course of atypical pneumonia. Note nonspecific interstitial pneumonia and a patchy infiltrate delineated by a feathery outline.

Genitourinary tract infections associated with the mollicutes

Although the mollicutes do not cause vaginitis, *M. hominis*, *M. genitalium*, and *U. urealyticum* are associated with infections of the urogenital tract. *M. genitalium* and *U. urealyticum* probably play a role in **nongonococcal urethritis (NGU)**. *M. hominis* and *U. urealyticum* are frequently isolated from asymptomatic sexually active individuals, making interpretation of a positive culture difficult. *U. urealyticum* has been recovered from more than 60% of healthy sexually active females. Because these species are opportunistic pathogens, the immune status of the host is an important factor in the

occurrence and severity of disease. In addition, it has been reported that among sexually active individuals, the rate of colonization is directly related to the number of sexual partners. Higher rates of colonization have been noted in adults of lower socioeconomic status.

M. hominis is found in the lower genitourinary tracts of approximately 50% of healthy adults but has not been reported as a cause of NGU. The organism may, however, invade the upper genitourinary tract and cause salpingitis, pyelonephritis, pelvic inflammatory disease (PID), or postpartum fever. It might also play a role in bacterial vaginosis. *Ureaplasma parvum* (*U. urealyticum* biovar 1) and *U. urealyticum* (*U. urealyticum* biovar 2) do not cause disease in the female lower genital tract. However, *U. urealyticum* has been associated with NGU in men, whereas *U. parvum* has not, although recent studies have found that both serovars often occur together, and horizontal gene transfer between the two species is common. Therefore maintaining separate serovars might not be valid. Evidence also does not support the role of the *Ureaplasma* spp. causing upper female genitourinary tract disorders.

While somewhat controversial, the urogenital mollicutes have also been linked to female infertility. Most females with infertility suffer from inflammation of the oviduct and nearby tissue. *Chlamydia trachomatis* and *Neisseria gonorrhoeae* are the most common causes of this condition, although other infectious agents have been implicated. The mollicutes can be part of the normal microbiota of the vagina and cervix. However, they can ascend and colonize the endometrium, fallopian tubes, and ovaries and cause inflammation and damage to the ciliated epithelium of the human fallopian tube. This can lead to scarring and infertility.

Ureaplasma spp. and *M. hominis* can be transmitted to neonates during delivery and have been associated with chorioamnionitis, congenital bacteremia, and pneumonia, as well as development of chronic lung disease in premature infants. Although it is not a primary cause of chronic lung disease, *U. urealyticum* is a common organism isolated from tracheal aspirates of low-birth-weight infants with respiratory disease; 14% of infections were in newborns delivered by cesarean section, thus indicating that infection occurred in utero and not during passage through the birth canal. *M. hominis* and *Ureaplasma* spp. have been recovered from the CSF of certain high-risk newborns, including preterm and low-birth-weight infants. Table 25.4 summarizes the known association of genital mollicutes with urogenital and newborn diseases. It has been recommended that culture for these organisms be attempted when the CSF specimen from a newborn with evidence of meningitis is negative for bacteria on Gram stain and routine bacteriology culture. However, because healthy neonates can be asymptotically colonized with mollicutes, routine screening cannot be justified.

Mycoplasma genitalium, first isolated in 1980, has been associated with NGU, cervicitis, endometriosis, and PID. There is also evidence linking *M. genitalium* to some cases of tubal sterility. Its prevalence is unknown, but PCR assays found *M. genitalium* more frequently in urethral samples taken from men with acute NGU than in those from men without urethritis. It is estimated that *M. genitalium* could be responsible for 30% of persistent urethritis in males.

Case check 25.1

In the Case in Point, CSF was culture negative for the more common causes of neonatal meningitis: group B *Streptococcus*, *Escherichia coli*, and *Listeria monocytogenes*. Both *M. hominis* and *U. urealyticum* have been isolated from the CSF of low-birth-weight infants. Because of the absence of a cell wall, these bacteria are not visible with Gram stain, so the lack of bacteria seen in direct Gram-stained smear supports the diagnosis.

In immunocompromised individuals, bacteremia and invasive disease of the joints and respiratory tract caused by mollicutes species have occurred. *U. urealyticum* has been reported to cause chronic inflammatory diseases, such as arthritis and cystitis, in patients with hypogammaglobulinemia. *Mycoplasma* isolates have been intermittently associated with patients with endocarditis, sternal wound infections, and arthritis.

Mycoplasma fermentans

Mycoplasma fermentans has been noted as a likely opportunistic respiratory pathogen. It is not known how often *M. fermentans* occurs in the respiratory tracts of healthy children, but it has been detected in throats of patients with lower respiratory tract infection, in some of whom a specific causative agent was not identified. Other groups of patients from whom *M. fermentans* has been recovered include adult patients with respiratory illness and those with acquired immunodeficiency syndrome (AIDS). *M. fermentans* has been isolated from tissue in patients with and without AIDS who died of systemic infection. *M. fermentans* has also been isolated from synovial fluid of patients with rheumatoid arthritis.

Laboratory diagnosis

Because they lack a cell wall, the mollicutes will not be visible by Gram stain. A DNA fluorescent stain (e.g., acridine orange) can be used, but this is not specific for the mollicutes. Laboratory diagnosis is often based on a nucleic acid amplification test (NAAT), culture, and serology. Immunochromatographic tests were released commercially in the 1980s. However, the assays demonstrated low sensitivity and specificity and are not recommended.

Specimens for culture and nonculture detection of the mollicutes include blood, synovial fluid, amniotic fluid, urine, prostatic secretions, semen, bronchoalveolar lavage fluid, as well as swabs from the nasopharynx, throat, cervix, and urethra. If swabs are used, it is important to vigorously rub the tissue because the mollicutes are strongly cell associated. Tissue samples may also be submitted for culture.

Specimen collection and transport

The absence of a cell wall makes all mollicutes extremely sensitive to drying and heat. Ideally, specimens should be inoculated at the bedside. If this is not possible, specimens should be delivered immediately to the laboratory in a transport

Table 25.4 Summary of association of genital mollicutes with urogenital and newborn diseases

Disease, target population	<i>Mycoplasma hominis</i>	<i>Mycoplasma genitalium</i>	<i>Ureaplasma</i> spp.	Comments
Nongonococcal urethritis	None	Weak	Strong ^a	<i>Ureaplasma</i> spp. cause some cases, but the proportion is unknown.
Prostatitis	None	Weak	None	An association with a few cases of chronic disease has been reported; a causal relation is unproven.
Epididymitis	None	None	Weak	<i>Mycoplasma</i> spp. are not an important cause.
Vaginitis and cervicitis	None	None	None	<i>M. hominis</i> is often associated with disease, but a causal relation is unproven.
Pelvic inflammatory disease	Strong	Strong	None	<i>M. hominis</i> causes some cases, but the proportion is unknown.
Postpartum fever	Strong	None	Weak	Recent studies indicate that <i>M. hominis</i> may be a major cause.
Urinary calculi	None	None	Weak	<i>Ureaplasma</i> spp. cause calculi in male rats, but no convincing evidence exists that they cause natural human disease.
Pyelonephritis	Strong	None	None	<i>M. hominis</i> causes some cases.
Involuntary infertility	Weak	Weak	Weak	<i>Ureaplasma</i> spp. are associated with altered motility of sperm.
Chorioamnionitis	Strong	None	Strong	An association exists, but a causal relation is unproven.
Low birth weight	None	None	Strong	An association exists, but a causal relation is unproven.
Neonatal infections, including sepsis, pneumonia, meningitis	Strong	None	Strong	Further clarification is needed, but importance is growing in a selected prenatal population.
Neonatal period, particularly preterm delivery, very low birth weight; clinical signs compatible with meningitis (CSF), pneumonia (trachea), sepsis (blood)	Strong	Weak	Strong	These findings need further clarification because most neonatal infections resolve without therapy, but in low socioeconomic groups, the diagnostic workup of newborns should include CSF and blood cultures for detection of mycoplasmas. This includes low-birth-weight and preterm newborns, in whom traditional CSF cell counts and cultures would be negative.

CSF, Cerebrospinal fluid.

^a*U. urealyticum* has been implicated in nongonococcal urethritis, while *U. parvum* has not been implicated.

medium, such as SP4 (sucrose phosphate buffer, *Mycoplasma* base, horse serum [20%], and neutral red) or Shepard 10B broth or 2SP, which are designed for *Mycoplasma*. Cotton-tipped swabs and wooden shafts should be avoided because of possible inhibitory effects. Swabs should be made of Dacron polyester or calcium alginate with aluminum or plastic shafts, and the swabs should be removed when the sample is placed in a transport medium. On arrival in the laboratory, the specimens should be frozen at -70°C if plating within 24 hours is not possible.

Nucleic acid amplification tests

Real-time PCR assays are the preferred method for the detection of many mollicutes. NAATs have the advantage of detecting bacteria earlier in the course of infection than serology, are more sensitive than serology, and have a rapid turnaround time. PCR assays can also differentiate *Ureaplasma* spp. A drawback to NAATs is that they cannot distinguish between

asymptomatic carriage and acute infection. Patients with *M. pneumoniae* infections can persistently harbor the organism for various lengths of time after the acute infection. Therefore it is difficult to interpret a positive PCR result. NAATs are less valuable for the more rapidly growing mollicutes, *M. hominis* and *Ureaplasma* spp., than the more difficult to isolate *M. pneumoniae* and *M. genitalium*. At least four NAATs for mollicutes have U.S. Food and Drug Administration approval. The BioFire Diagnostics (Salt Lake City, UT) FilmArray RP (respiratory panel) detects nucleic acid from 14 respiratory pathogens, including *M. pneumoniae*, in nasopharyngeal swabs.

Culture

Because recovery from culture is difficult (sensitivity approximately 40%), isolation of *M. pneumoniae* from respiratory sites is infrequently attempted. Growth may take several weeks, and technical expertise is necessary. *M. hominis* and *Ureaplasma* spp. are less stringent in their growth requirements

Table 25.5 Major clinical and corresponding diagnostic manifestations of *Mycoplasma pneumoniae*

Manifestation	Days after onset						
	5	10	15	20	25	35	40
Headache and malaise	+ 1	+ 3	+ 3	+ 2	+ 1		
Dry cough	+ 2	+ 4	+ 4	+ 1			
Chest soreness	+ 3	+ 3	+ 1				
Fever							
With antimicrobial treatment	104° F	104° F	102° F	100° F	Absent		
Without antimicrobial treatment	104° F	100° F	Absent	Absent	Absent		
Chest radiography	+ 2	+ 3	+ 2	+ 2	+ 1		
<i>Mycoplasma</i> culture with or without antimicrobial treatment	+	+	+	+	+	+	
Complement fixation (titer)	≤8	8	32	64	256	256	128
<i>Mycoplasma</i> -specific Ig							
IgM	–	+	+	+	+	+	+
IgG	–	–	+	+	+	+/–	–

+ 4, Most severe; + 1, least severe; +, present or positive; –, absent or negative; IgG, immunoglobulin G; IgM, immunoglobulin M.

but require cholesterol for synthesis of plasma membranes. *M. hominis* is the only species that will grow on sheep blood and chocolate agars. Diagnosis of *M. pneumoniae* infection is usually established serologically, traditionally with acute-phase and convalescent sera collected 2 to 3 weeks apart to demonstrate a fourfold increase in titer. A representation of classic clinical and corresponding diagnostic manifestations of *M. pneumoniae* is shown in Table 25.5. As noted, many of the early symptoms are nonspecific, and a thorough understanding of the disease process is necessary for interpretation of serum and culture results.

Media

Several media have been developed for the recovery of mollicutes, and no single medium is suitable for all species isolated from humans. Penicillin can be added to minimize bacterial contamination. *M. hominis* and *Ureaplasma* spp. are more rapid growers and relatively easy to recover compared with the other mollicutes. SP4 broth and agar are ideal for *M. pneumoniae* and *M. hominis*. *M. pneumoniae* and *M. genitalium* require glucose (their major energy source), *M. hominis* requires arginine, and *Ureaplasma* spp. require urea. *Ureaplasma* spp. also require media to have a pH near 6.0 (Shepherd 10B arginine broth) with a buffer to maintain the pH. It is difficult to sustain *Ureaplasma* spp. in culture because death occurs rapidly when the urea is depleted, and the bacteria are sensitive to changes in pH because of urea utilization. *M. hominis* and *U. urealyticum* require cholesterol for synthesis of plasma membranes and other undetermined growth factors; fetal calf serum (20% vol/vol) is the traditional nutrient source. A8 agar can be used as a solid medium to recover *M. hominis* and *Ureaplasma* spp. Because mycoplasmas do not produce turbidity in broth media, a pH indicator, such as phenol red, should be added to detect growth.

Recovery of mycoplasma from blood can be performed by placing uncoagulated blood into mycoplasmal broth media. A ratio of 1:10 (blood to broth) and 10 mL of blood for adults is recommended. Sodium polyanethol sulfonate (SPS), an

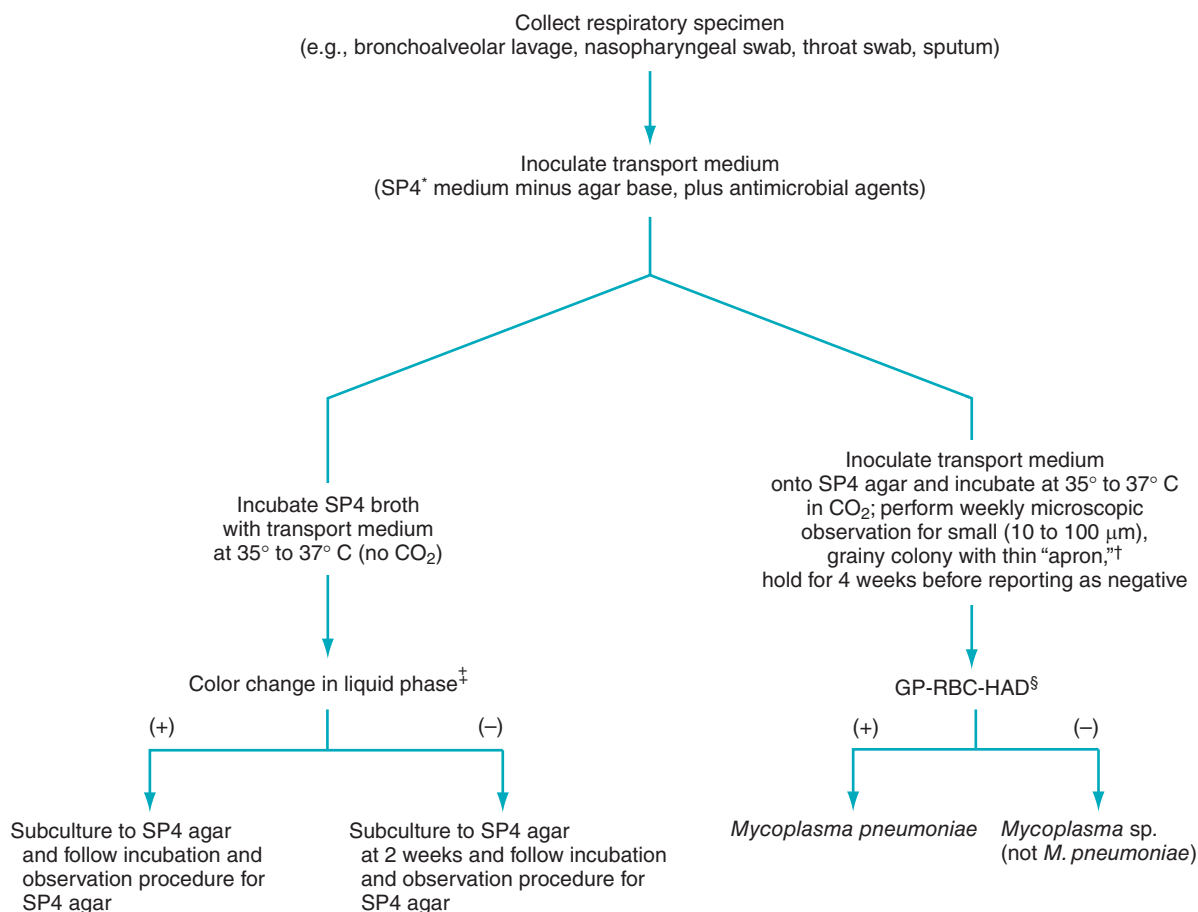
additive often found in commercial blood culture media, is inhibitory to mycoplasma. The addition of 1% (weight per volume) gelatin might help overcome the inhibitory effect of SPS. Nevertheless, the use of commercial blood culture media, whether or not used in automated instruments, is not recommended. Fig. 25.5 presents a schematic representation of media and methods used in the traditional procedures for isolation and identification of *Mycoplasma* spp.

Fluids should be centrifuged and the pellet resuspended in a small volume of liquid for inoculation of media. It is important that specimens be diluted in broth up to 10⁻³ before each dilution is plated. This helps minimize the inhibitory effects of antimicrobial agents, antibodies, and other antibacterial factors that might be present in the specimen.

Commercial culture media and kits for the detection and recovery of mollicutes have been developed and are available in the United States and Europe. Such products may detect, quantify, identify, and determine the antimicrobial susceptibility of genital mycoplasmas from urogenital specimens and *M. pneumoniae* from respiratory secretions. These kits are useful in laboratories that infrequently perform cultures for the mollicutes, but laboratory scientists must be aware of the limitations of these assays and perform internal quality control.

Isolation and identification

Once inoculated, broth media should be placed at 35° C under atmospheric conditions, whereas solid agar media may be incubated in an environment of room air enhanced with 5% to 10% carbon dioxide (CO₂) or in an anaerobic atmosphere of 95% nitrogen gas (N₂) with 5% CO₂. *M. hominis* and *Ureaplasma* spp. colonies may appear within 2 to 4 days, whereas *M. pneumoniae* colonies may take 21 days or longer. *Mycoplasma*-like colonies are stained with Dienes or methylene blue stain. Staining is performed by placing a small block of the agar on a glass slide, covering the colony with the stain, adding a coverslip, and examining the agar microscopically under low power. *M. hominis* has a typical “fried egg” appearance, with



*SP4 is sucrose phosphate buffer, Mycoplasma base, fetal bovine serum (20%), phenol red. Medium stabilizes and decontaminates specimen. Storage at -70°C for repeated testing is recommended.

†Thin colony periphery. Examine with stereomicroscope using $\times 20$ to $\times 60$ magnification.

‡Color change: positive, yellow color with no gross turbidity; negative, red color.

§Guinea pig red blood cell hemadsorption (GP-RBC-HAD). β -Hemolysis test for presumptive identification of *Mycoplasma pneumoniae* may be used in lieu of GP-RBC-HAD.

Note: Methylene blue or Dienes stain can be used for detection of *Mycoplasma* spp. on SP4 agar; plate immunofluorescence using labeled antibody and nucleic acid amplification can be used for identification.

Fig. 25.5 Flow diagram for *Mycoplasma* spp. isolation using classic traditional methods.

the periphery staining a light blue and the center dark blue (Fig. 25.6). *Mycoplasma* spp. almost universally show a mixed colony presentation on primary isolation when examined with a stereomicroscope (Fig. 25.7).

Ureaplasma spp., once called **T-strain mycoplasma** (*T* for "tiny"), form extremely small colonies that are difficult to see with the naked eye; hence, mycoplasmal cultures on solid media should always be examined with a stereomicroscope. Fig. 25.8 shows *M. hominis* and *U. urealyticum* grown on New York City agar. Urease activity of *Ureaplasma* may be detected on solid agar containing urea and manganese chloride (U9B urease color test medium). Urease-positive colonies are a dark golden-brown color because of the deposition of manganese dioxide.

Although uncommon, extragenital *M. hominis* infections are emerging. This organism should be considered whenever many polymorphonuclear cells are seen on Gram stain, but

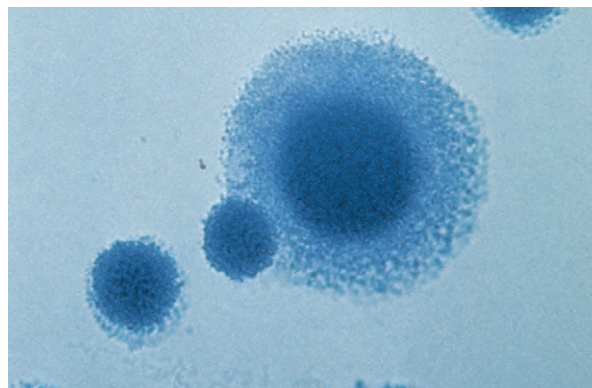


Fig. 25.6 Dienes stain of *Mycoplasma* spp. colonies demonstrating typical fried egg appearance ($\times 40$).

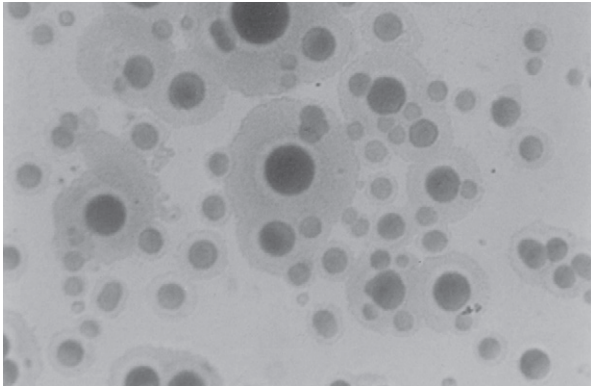


Fig. 25.7 Typical mixed sizes of *Mycoplasma* spp. on primary isolation media, *Mycoplasma salivarium* ($\times 20$). (Courtesy Bionique Testing Laboratories, Saranac Lake, NY)

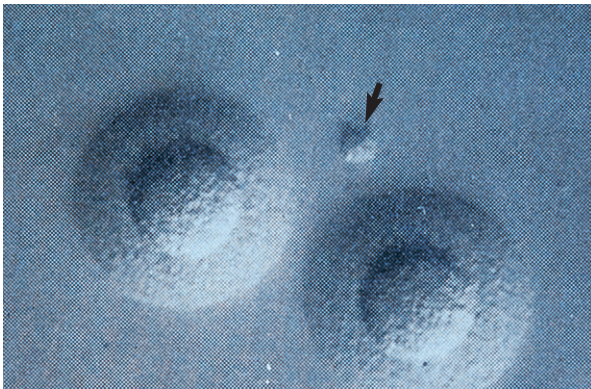


Fig. 25.8 Mixed isolation of *Mycoplasma hominis* and *Ureaplasma urealyticum* showing why *U. urealyticum* was originally called T-strain mycoplasma, T for "tiny" (arrow) ($\times 40$).

there is no growth on routine bacterial culture. *M. hominis* grows well anaerobically and will appear as pinpoint (0.05-mm), clear, glistening, raised colonies on Columbia colistin-nalidixic acid agar or anaerobic blood agar (Centers for Disease Control and Prevention formula) in 48 hours. Under anaerobic conditions, the colonies do not display the "fried egg" morphology. The anaerobic plate should be examined by using oblique light. The colonies that do not take up Gram stain should be subcultured to A7 medium, on which they demonstrate typical "fried egg" growth and stain positive with Dienes or methylene blue stain if they are *Mycoplasma* spp.

Although not conclusive, growth rate, body site recovered from, and colony appearance can aid in the identification of *Mycoplasma*. Glucose utilization in SP4 broth will cause an acid shift producing a yellow color, whereas arginine metabolism will produce an increase in pH, changing the indicator to a deeper red color. In 10B broth, urea or arginine utilization will increase the pH, changing the pH indicator from orange to deep red. A slow-growing mycoplasma from a respiratory specimen producing a yellow color in SP4 broth is likely *M. pneumoniae*. Production of an alkaline reaction in 10B broth after overnight incubation of a urogenital specimen is suggestive of *U. urealyticum*, whereas an alkaline shift in media with arginine within 24 to 72 hours is likely caused by *M. hominis*.

Case check 25.2

In the Case in Point, an infection caused by *U. urealyticum* would produce an alkaline shift in media containing urea in about 24 hours. If the infection was caused by *M. hominis*, an alkaline shift would occur in media containing arginine in 24 to 72 hours.

Previously, identification of the mollicutes was often done by typing methods using monoclonal antibodies and immunofluorescence and observation of plate media with a stereomicroscope. A direct plate immunofluorescent method also can be used. Fluorescent-labeled, anti-*M. pneumoniae* antibody is flooded on colonies on the plate; the plate is then washed and examined for immunofluorescence. The characteristic of guinea pig red blood cells (0.4% in saline) adhering to colonies of *M. pneumoniae* and not *M. hominis* is another standard assay that helps distinguish the two species. Furthermore, guinea pig cells do not adhere to large-colony *Mycoplasma* spp., which are common inhabitants of the upper respiratory tract. PCR-based assays are now commonly used because they are widely available, less subjective, and easier to perform. Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry was shown to identify most human mollicute species. However, testing requires 30 mL to 100 mL of culture for the mollicutes, limiting the use of this method.

Serologic diagnosis

Because of the inherent difficulties of cultures and interpretations of a positive PCR assay result, *M. pneumoniae* has historically been diagnosed by serologic methods. Optimally, serum samples for serologic testing should be collected at the onset of symptoms and 2 to 3 weeks later for acute-phase and convalescent measurements. This delay in testing is an obvious drawback. However, using a single immunoglobulin M (IgM) determination is flawed. Some individuals, especially those older than 40 years of age, might not produce IgM, possibly due to previous exposure. In addition, IgM antibody can persist for weeks to months.

M. pneumoniae induces the formation of autoantibodies that bind to a number of antigens on host cells, including the I antigen found on human red blood cells. Because they agglutinate type O Rh-negative erythrocytes at temperatures below normal body temperature (optimal is 4° C), anti-I antibodies are referred to as *cold agglutinins*. The cold agglutinin antibody titer was used for many years as an indicator of primary atypical pneumonia, but it is insensitive and nonspecific for *M. pneumoniae*. Approximately 50% of patients with primary atypical pneumonia produce a detectable cold agglutinin antibody titer. This assay is no longer recommended for the diagnosis of *M. pneumoniae* infection.

Previously, the most used technique for demonstration of *M. pneumoniae*-specific antibodies was the complement fixation assay, which was time-consuming and had inherent technical problems. The assay also suffered from false-positive results caused by autoantibodies and cross-reactivity to *M. genitalium*. Several commercially available enzyme immunoassays and immunofluorescence assays are now available for the detection of serum antibodies and, in some cases,

detect IgM or IgG. Table 25.6 highlights selected features of these immunologic assays and other methods. It is important to remember that demonstration of a significant increase in antibody titer in conjunction with culture isolation is preferable for a definitive diagnosis. Serologic methods are available for *M. hominis* and *U. urealyticum* but are generally performed only by reference laboratories and are not recommended for routine diagnosis.

Antimicrobial susceptibility

The mollicutes are inherently resistant to the β -lactams (penicillins and cephalosporins) as well as sulfonamides, trimethoprim, and rifampin because they lack a cell wall. *M. pneumoniae* has remained susceptible to the tetracyclines, newer fluoroquinolones, and the macrolides (e.g., erythromycin). However, there have been scattered reports of high-level macrolide resistance. Roughly 15% of United States isolates are macrolide resistant. Because of side effects, tetracycline is used only for the treatment of adults. *M. hominis*, which is more resistant than *M. pneumoniae*, is usually resistant to erythromycin but susceptible to clindamycin and lincomycin, whereas *U. urealyticum* is generally resistant to clindamycin and lincomycin and susceptible to erythromycin. Both organisms are often susceptible to tetracycline, but high-level resistance is emerging and is common in some geographic areas.

Standard minimal inhibitory concentration methods for susceptibility testing by broth microdilution and agar dilution of mycoplasma have been established by the Clinical and Laboratory Standards Institute. The agar dilution method is regarded as the reference method; however, because of the high degree of technical expertise required and the few mollicute isolates, this assay is not offered by most hospital laboratories. The broth microdilution is the most used method to determine the minimal inhibitory concentration. With antimicrobial resistance increasing, the availability of newer broad-spectrum antimicrobials, and the emergence of more

infections caused by *Mycoplasma* spp., antimicrobial susceptibility testing methods are becoming more important. Because of the variable susceptibility pattern of *M. hominis*, antimicrobial susceptibility testing is usually recommended for clinically significant isolates. These isolates should be forwarded to a reference laboratory for testing.

Historically, *M. pneumoniae* had a predictable sensitivity pattern, so antimicrobial susceptibility testing was not often warranted. However, with high-level macrolide resistance increasing, molecular assays were developed to detect mutations in 23S rRNA that results in macrolide resistance. These assays can be performed directly on clinical specimens eliminating the need for cultures to isolate the organism.

Interpretation of laboratory results

M. pneumoniae detected by any method from pulmonary or nonpulmonary specimens should be considered significant and a pathogen. The high sensitivity of the PCR assay means that a positive result must be correlated with the clinical picture. Interpretation of *M. hominis* isolation is not as obvious; differentiation from colonization and infection requires detailed clinical analysis and potentially repeated cultures. Isolation from a normally sterile site is significant.

U. urealyticum is the most difficult to assess clinically. In urogenital specimens, it has been reported to colonize up to 70% of men and 45% of women with no apparent infection. Its isolation is not indicative of pathogenicity, and it is incumbent on the laboratory to educate the health care provider. Culture results should include a statement suggesting its potential for colonization versus pathogenicity. In these specimens, quantification is important. In sterile specimens, particularly CSF isolates, it is reasonable to assume that isolation is significant.

Respiratory specimens received in the laboratory often provide limited clinical information. Specimens are processed and inoculated onto the appropriate media given the

Table 25.6 Comparative features of various laboratory methods used to detect *Mycoplasma pneumoniae*, *Mycoplasma hominis*, and *Ureaplasma urealyticum*

Detection method	<i>Mycoplasma pneumoniae</i>	<i>Mycoplasma hominis</i>	<i>Ureaplasma urealyticum</i>
Nonserologic			
Culture	Traditionally difficult	Method of choice, but must differentiate infection from colonization	Method of choice using urease detection, but must differentiate infection from colonization
Polymerase chain reaction	Commonly used	Occasionally used	Occasionally used
Serologic			
Complement fixation	Traditional assay, but need to demonstrate fourfold increase between acute-phase and convalescent sera; >32 single titer might be suggestive	Not recommended	Not recommended
Immunofluorescent antibody	Separately measures IgG and IgM	Not recommended	Not recommended
Enzyme immunoassay	Separately or simultaneously measures IgG and IgM	Not recommended	Not recommended

IgG, Immunoglobulin G; IgM, immunoglobulin M.

Table 25.7 Laboratory detection of frequent respiratory pathogens

Epidemiologic factors				Laboratory methods			
Age	Organism Frequently Involved	Disease	Season	Specimen Source	Stain	Culture	Nonculture
Newborn	1, 3, 4, 7	Pneumonia	Year round ^a	Tracheal suction	Gram	Routine, plus mycoplasmal	
Grade school	2, 4	Atypical pneumonia	Fall and winter	Sputum, nasopharynx	Gram	Routine, plus mycoplasmal	Mycoplasma serology
College student	1, 2, 8	Biphasic disease with pharyngitis and later, bronchitis	Year round ^b	Sputum, nasopharynx	None ^c	<i>B. pertussis</i>	Mycoplasma serology
Adult	2, 4, 5, 6	Pneumonia or atypical pneumonia	Year round ^a	Sputum	Gram	Routine	Mycoplasma serology
				Bronchoalveolar lavage specimen	Gram, Gomori methenamine silver, and/or calcofluor white	Routine, plus fungal	

1, *Chlamydia pneumoniae*; 2, *Mycoplasma pneumoniae* (outbreak); 3, *Mycoplasma hominis*; 4, viral, e.g., adenovirus, respiratory syncytial virus, influenza virus (seasonal); 5, other—fungus, *Legionella*, or *Pneumocystis pneumoniae*; 6, *Streptococcus pneumoniae*; 7, *Streptococcus agalactiae*; 8, *Bordetella pertussis*.

^aSeasonal incidence depends on the pathogen.

^bThe greatest seasonal incidence of pertussis is spring and summer, followed by winter.

^cIn place of direct stains, nucleic acid amplification tests for *C. pneumoniae* and/or *B. pertussis* should be performed.

most likely candidate for the disease, clinical presentation, age of the patient, and seasonality, recognizing that there is a certain predictability with selected pathogens. Table 25.7 presents laboratory methods used to diagnose infections caused by several pathogens—*Mycoplasma*, *Chlamydia*, *Legionella*, mycobacteria, fungi, and viruses—in various age groups. All respiratory specimens should be stored at -80°C if storage time is likely to exceed 24 hours. Acute-phase sera should also be stored frozen for subsequent antibody titer testing.

POINTS TO REMEMBER

- The mollicutes are minute organisms characterized by the lack of a cell wall. Because of the lack of a cell wall, the mycoplasmas are inherently resistant to the β -lactam antibiotics.
- The most clinically significant species of the Mycoplasmataceae are *M. pneumoniae*, *M. hominis*, *M. genitalium*, *M. fermentans*, and *Ureaplasma* spp., although others are beginning to be recognized as opportunistic pathogens.
- M. pneumoniae* is an important cause of community-acquired, atypical pneumonia. It is not considered part of the normal human microbiota.
- M. hominis*, *M. genitalium*, and *Ureaplasma* spp. are genital mollicutes.
- Because of the difficulty to isolate, *M. pneumoniae* infection is most often diagnosed by serologic methods. *M. hominis* and *Ureaplasma* spp. are commonly diagnosed by culture, although PCR technology is also available. *M. genitalium* and *M. fermentans* are generally detected by PCR.

LEARNING ASSESSMENT QUESTIONS

- From which source did the neonate described in the Case in Point likely acquire the infection?
 - Breast milk
 - Inhalation after birth
 - Passed through the birth canal
 - Infectious agent crossed the placenta
- Why was the Gram stain result negative in the Case in Point?
 - Mollicutes do not Gram stain.
 - The Gram stain is too insensitive.
 - Mollicutes do not retain safranin; need to use a different counterstain.
 - When mollicutes are suspected, the time needed to heat fix the smear must be extended.
- How does primary atypical pneumonia caused by *M. pneumoniae* differ from pneumonia caused by *S. pneumoniae*?
 - Has a lower mortality rate
 - Is not spread person to person
 - Is seen more often in older adults
 - Is characterized by a bloody, productive cough
- Which special stain is used on suspected colonies of *Mycoplasma*?
 - Acridine orange
 - Calcofluor white
 - Dienes
 - Silver
- An amniotic fluid is submitted to the microbiology laboratory for isolation of mycoplasma. It will be 48 hours before the specimen can be processed. At what temperature should the specimen be stored?

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Mycobacterium tuberculosis and nontuberculous mycobacteria

Donald C. Lehman

CHAPTER OUTLINE

General characteristics, 571

Clinical significance of the *Mycobacterium tuberculosis* complex, 572

Mycobacterium tuberculosis, 572

Mycobacterium bovis, 575

Clinical significance of nontuberculous mycobacteria, 575

Slowly growing species, 575

Rapidly growing species, 579

Mycobacterium leprae, 580

Isolation and identification of mycobacteria, 580

Laboratory safety considerations, 583

Specimen collection, 583

Digestion and decontamination of specimens, 585

Concentration procedures, 586

Staining for acid-fast bacilli, 586

Culture media and isolation methods, 587

Laboratory identification, 589

Susceptibility testing of *Mycobacterium tuberculosis*, 594

Bibliography, 596

OBJECTIVES

After reading and studying this chapter, you should be able to:

1. Compare the general characteristics of mycobacteria with those of other groups of bacteria.
2. Discuss the clinical disease caused by *Mycobacterium tuberculosis*.
3. Analyze patient results obtained with the tuberculin skin test and interferon-gamma release assay for the diagnosis of tuberculosis.
4. Develop a protocol for the isolation and identification of *M. tuberculosis* from a sputum specimen.
5. Discuss the clinical significance of nontuberculous mycobacteria.
6. Describe the appropriate specimen collection and processing procedures to recover mycobacteria from clinical samples.

7. Discuss the safety precautions to be followed while working in a mycobacteriology laboratory.
8. Justify the digestion and decontamination requirements of certain clinical specimens for the isolation of mycobacteria.
9. Describe the principles and procedures for the stains used to demonstrate mycobacteria in clinical samples and culture isolates.
10. Compare the different culture media used for the isolation of mycobacteria.
11. Discuss the different tests used to identify mycobacteria.
12. Compare continuous monitoring systems with those of conventional media for detecting mycobacterial species in clinical samples.

KEY TERMS

Acid fastness

Auramine stain

Auramine-rhodamine fluorochrome stain

Granuloma

Kinyoun stain

Middlebrook 7H11 medium

Miliary tuberculosis

Mycobacterium avium complex (MAC)

Mycobacterium tuberculosis complex (MTBC)

Nonchromogenic

Nontuberculous mycobacteria (NTM)

Photochromogenic

Pott disease

Purified protein derivative (PPD)

Scotochromogenic

Ziehl-Neelsen stain

Case in point

A 56-year-old male patient came to the emergency department complaining of fatigue and weight loss (10 lb) over the past 12 months. The patient also complained of a cough for 3 months that produced red-tinged sputum. He indicated a history of night fever and chills but reported not having dyspnea or chest pain. The patient, who moved to the United States from Mexico, had a family history of pulmonary tuberculosis. He reported that his last purified protein derivative (PPD) skin test, performed approximately 5 years ago, was nonreactive. Vital signs included temperature of 36.5° C (97.7° F), pulse of 63 beats per minute, respirations of 15 breaths per minute, and

blood pressure of 96/56 mm Hg. Chest radiography revealed an infiltrate in the upper lobe of the right lung. Computed tomography of the chest showed a nodular patchy opacity in the upper lobe of the right lung. The patient was admitted for further evaluation. A PPD skin test showed a 10-mm by 7-mm induration. Three sputum samples were obtained over a 3-day period for acid-fast bacilli (AFB) smear and culture. All three samples were reported as no AFB seen on direct acid-fast stained smears. Processed samples were inoculated onto Löwenstein-Jensen (LJ) medium and into BACTEC 12B bottles (BD Diagnostic Systems, Sparks, MD). After 12 to 14 days of incubation, the BACTEC bottles from all three specimens were positive. Kinyoun staining of smears from the bottles revealed AFB. Polymerase chain reaction (PCR) DNA amplification for *Mycobacterium tuberculosis* of the BACTEC medium showed a positive result. A four-drug antituberculosis regimen comprising isoniazid, rifampin, pyrazinamide (PZA), and ethambutol was recommended.

Issues to consider

After reading the patient's case history, consider:

- Significant aspects of this patient's family history
- The characteristic symptoms of tuberculosis
- The typical length of time for a culture to yield pathogenic *Mycobacterium*

The genus *Mycobacterium* is composed of over 180 recognized and proposed species. The most familiar of the species are *Mycobacterium tuberculosis* and *Mycobacterium leprae*, the causative agents of tuberculosis (TB) and Hansen disease (leprosy), respectively. Both diseases have long been associated with chronic illness and social stigma. TB remains a major cause of morbidity and mortality in the world today and is the leading cause of death resulting from a single infectious agent—surpassing human immunodeficiency virus (HIV). In 2020, The *Global Tuberculosis Report* by the World Health Organization (WHO) estimated 9.9 million new cases, and 1.5 million people died from TB, including 214,000 people with HIV worldwide.

Nontuberculous mycobacteria (NTM)

In addition to TB and Hansen disease, *Mycobacterium* spp. produce a spectrum of infections in humans and animals. A large group of mycobacteria, excluding the *M. tuberculosis* complex (MTBC) and *M. leprae*, normally inhabit the environment and can cause disease that often resembles TB in humans. These organisms are sometimes referred to as *atypical mycobacteria*, **nontuberculous mycobacteria (NTM)**, or *mycobacteria other than the tubercle bacillus (MOTT)*. The term *nontuberculous mycobacteria* is used here. The growing number of immunocompromised individuals worldwide has led to a resurgence of TB and diseases caused by NTM. Box 26.1 shows the usual clinical significance of *Mycobacterium* spp. isolates.

Epidemiologic changes have led to challenges in the mycobacteriology laboratory, including rapid identification of all clinically significant mycobacteria and antimicrobial susceptibility testing of *Mycobacterium* spp. Fortunately, new developments in the field of clinical mycobacteriology are helping meet these challenges. Rapid methods, like nucleic acid amplification tests (NAATs), can eliminate the need for lengthy culturing and protracted biochemical methods of identification. Further developments in the application of molecular biology, including matrix-assisted laser desorption/ionization–time-of-flight (MALDI-TOF) mass spectrometry

BOX 26.1 Usual clinical significance of *Mycobacterium* species isolates

Pathogen

Mycobacterium tuberculosis
Mycobacterium bovis
Mycobacterium africanum
Mycobacterium ulcerans
Mycobacterium leprae

Often pathogen, potential pathogen

Mycobacterium avium complex
Mycobacterium kansasii
Mycobacterium marinum
Mycobacterium haemophilum
Mycobacterium genavense
Mycobacterium abscessus subsp. *abscessus*
Mycobacterium abscessus subsp. *massiliense*
Mycobacterium abscessus subsp. *bolletii*
Mycobacterium chelonae
Mycobacterium fortuitum group
Mycobacterium simiae
Mycobacterium szulgai
Mycobacterium xenopi

Potential pathogen

Mycobacterium mageritense
Mycobacterium scrofulaceum

Usual saprophyte, rare pathogen

Mycobacterium goodii
Mycobacterium flavescens
Mycobacterium gastri
Mycobacterium nonchromogenicum
Mycobacterium terrae
Mycobacterium phlei
Mycobacterium smegmatis
Mycobacterium vaccae

ionization–time-of-flight (MALDI-TOF) mass spectrometry for identification, increase accuracy and reproducibility, ease performance, and reduce cost.

General characteristics

Mycobacteria are slender, slightly curved or straight, rod-shaped organisms 0.2 to 0.6 μm $\times$ 1 to 10 μm in size. They are nonmotile and do not form spores. However, limited studies claim to demonstrate spores or sporelike structures in *Mycobacterium marinum*. The cell wall has extremely high lipid (mycolic acid) content; thus mycobacterial cells resist staining with commonly used basic aniline dyes, such as those used in Gram stain, at room temperature. Mycobacteria take up dye with increased staining time or application of heat but resist decolorization with acid-ethanol. This characteristic, referred to as acid fastness—hence the term *acid-fast bacilli* (AFB)—distinguishes mycobacteria from most other genera. Mycolic acids are also found in other genera (e.g., *Corynebacterium* and *Rhodococcus*); however, the amount of mycolic acids in the mycobacteria is much larger than in these other bacteria.

Mycobacteria are strictly aerobic, but increased carbon dioxide (CO₂) concentration will enhance the growth of some species. The pathogenic mycobacteria grow more slowly than most other bacteria that are pathogenic to humans. Typically, mycobacteria associated with disease require 2 to 6 weeks of incubation on complex media at specific optimal temperatures, whereas the rapidly growing species generally grow on simple media in 2 to 3 days at temperatures of 20° to 40° C. One of the mycobacteria pathogenic to humans, *M. leprae*, fails to grow in vitro.

Clinical significance of the *Mycobacterium tuberculosis* complex

The *Mycobacterium tuberculosis* complex (MTBC) consists of *M. tuberculosis*, *M. bovis* (including the vaccination strain bacillus Calmette-Guérin [BCG]), *M. africanum*, *M. microti*, *M. canettii*, *M. caprae*, *M. pinnipedii*, *M. mungi*, *M. orygis*, *M. mungi*, and *M. suricattiae*. Members of the MTBC display a high degree of genetic homogeneity, although they have different phenotypic characteristics and mammalian host ranges. Within this group, most human infections are caused by *M. tuberculosis* and to a lesser extent *M. bovis* and *M. africanum*. *M. bovis* is found primarily in cattle, but it can infect other mammals, including humans. *M. africanum* has been associated with human cases of TB in tropical Africa, and *M. microti* has been linked to TB in both immunocompetent and immunocompromised individuals. *M. canettii* has been reportedly causing infections in children and patients with HIV infection in Africa. The remaining species are infrequently associated with human infections.

Mycobacterium tuberculosis

Mycobacterium tuberculosis was first described by Robert Koch in 1882. However, TB is one of the oldest documented communicable diseases and remains a leading cause of morbidity and mortality globally. A disease of poverty, as many as 25% of the world's population may be infected with the bacteria causing TB. About 90% of TB cases occur in 30 TB high-burden countries. The geographic areas with the most cases are Southeast Asia (44%), Africa (25%), and the Western Pacific (18%). The United States has one of the lowest TB rates in the world. In 2021, 7860 TB cases were reported, 687 more than during 2020 (7173), a 9.4% increase in TB incidence (cases per 100,000 population) from 2.16 to 2.37. However, up to 13 million people living in the United States have latent TB infection. These individuals are infected with the bacteria causing TB but remain asymptomatic. Currently, about 71% of the cases are in foreign-born individuals from endemic areas.

Primary tuberculosis

After exposure to *M. tuberculosis*, whether or not a person develops TB is determined by his or her cellular immune response, the infective dose, and the virulence of the strain. TB is usually a disease of the lower respiratory tract. Typical signs and symptoms include malaise, weight loss, night sweats, and cough that can produce or not produce sputum that is with or without blood. Tubercle bacilli are acquired

from persons with active disease who are excreting viable bacilli by coughing, sneezing, or talking. Airborne droplets containing bacteria enter the respiratory tract of an exposed individual and reach the lung alveoli. *M. tuberculosis* cells are phagocytized by alveolar macrophages but can prevent fusion of phagosomes containing bacteria with lysosomes. This permits intracellular multiplication of the bacteria. In a person with adequate cellular immunity, macrophages secrete interleukin-12 and tumor necrosis factor alpha, which recruit T cells and natural killer cells and enhance the inflammatory reaction. Some of the T cells differentiate into T-helper cells type-1 (Th1) releasing lymphokines, such as interferon-γ (IFN-γ). IFN-γ stimulates macrophages in the infection site to destroy intracellular mycobacteria. This is followed by regression and healing of the primary lesion.

In many exposed individuals, the immune system does not initially eliminate the bacteria, and this can result in life-long or latent infections. The pathologic features of TB are the result of a hypersensitivity reaction to mycobacterial antigen. If there is little antigen and a strong hypersensitivity reaction, a small, hard tubercle, or granuloma, may be formed. The granuloma is an organization of lymphocytes, macrophages, fibroblasts, and capillaries, along with fibrosis encapsulation. With granuloma formation, the bacteria are contained and can ultimately be killed, and healing occurs along with calcification and scar formation as a reminder of the past infection.

If the antigen load and hypersensitivity reaction are both high, tissue necrosis from the enzymes of degenerating macrophages can occur; the tissue response is less organized, and granulomas are surrounded by fibrin that protects the bacteria from phagocytosis by macrophages. Necrotic or caseous material can be present at the site of the primary lesion because of solid or semi-solid amorphous material deposited at the site of necrosis. After healing of the primary infection, the bacilli are not totally eradicated but can remain viable, but dormant, in granulomas for months or years. In infected individuals, there is a potential for reactivation of TB when the bacteria are released from the granulomas. This happens because of immunosuppression from old age or other causes.

A small percentage of individuals who are infected with TB develop progressive (active) pulmonary disease, usually from a failed cellular immune response and, hence, a failure to stop multiplication of the bacilli. In young children or older adults with primary infection and in people with an underlying immunodeficiency, massive lymphohematogenous dissemination may occur and lead to meningeal or miliary (disseminated) TB, which is discussed later. In addition, 10% of young adults may progress to active disease from their primary infection. This will resemble reactivation TB in older adults; the only way to differentiate it is a positive purified protein derivative (PPD) skin test result in a previously negative individual.

Case check 26.1

A positive PPD skin test result only indicates past exposure to *M. tuberculosis*; it does not imply a recent infection. In the Case in Point, the patient reported a negative skin test result obtained about 5 years ago, thus establishing a negative baseline. During his current evaluation, he had a positive skin test result, indicating exposure and subsequent immune response at some point in the past 5 years.

Reactivation tuberculosis

Approximately 5% of those infected develop active disease within 5 years, and an additional 5% to 10% develop disease within their lifetime. Progression from infection to active disease differs, depending on the patient's age and the intensity and duration of exposure. Malnutrition, with or without other factors, such as alcoholism, homelessness, incarceration, immunosuppression, and HIV infection, can contribute greatly to progression to active TB. Reactivation TB also occurs more frequently in patients with **immune-mediated inflammatory diseases like systemic lupus erythematosus and rheumatoid arthritis**.

Reactivation TB from a latent infection occurs when there is an alteration or suppression of the cellular immune system in the infected host that favors replication of the bacilli and progression to disease. The symptoms of **disease are slow in developing (insidious) and consist of fever, shortness of breath, night sweats and chills, fatigue, anorexia, and weight loss**. About 20% of individuals may have no symptoms, but most patients eventually have productive cough, chest pain, and fever. Hemoptysis, indicating cavitation and necrosis, occurs in 25% of cases. In patients with reactivation TB, radiography reveals a patchy or confluent consolidation with increased linear densities extending to the hilum; thick-walled cavities without air-fluid levels usually are found in the apical or posterior segments of the upper lobe or in the superior segment of the middle lobe of the lung. If there is bronchogenic spread of the bacilli, multiple alveolar densities will be seen; rarely is there enlargement of the lymph nodes.

In chronic disease, fibrosis, loss of lung volume, and calcifications will be demonstrated. **The PPD skin test may yield negative results in up to 25% of these cases; diagnosis is confirmed by stained smear and culture of sputum, gastric aspirates, or bronchoscopy specimens**. Fiberoptic bronchoscopy has been found to yield 95% recovery of the bacteria; postbronchoscopic sputa are also usually positive. In any case of pulmonary TB, complications could occur if diagnosis and treatment are delayed. These include empyema, pleural fibrosis, massive hemoptysis, adrenal insufficiency (rare), and hypercalcemia (up to 25% of cases). In patients with acquired immunodeficiency syndrome (AIDS) and TB with drug-resistant bacilli, the risk of progression to disease from infection is quite high, although clinical findings may differ from those in the patient with AIDS and reactivation TB. The diagnosis is usually made by stained smears and culture, with a rate of sensitivity similar to that in patients without AIDS.

Extrapulmonary tuberculosis

Disseminated or extrapulmonary tuberculosis (EPTB), infection outside the lungs, occurred much less commonly than pulmonary TB before the AIDS epidemic. In the WHO's 2020 *Global Tuberculosis Report*, extrapulmonary TB represented 16% of the 7.1 million incident cases. **It has been suggested that an increased prevalence of risk factors for EPTB, such as HIV infection, foreign-born status, and advanced age of the population, has contributed to an increased** proportion of EPTB cases in the United States.

The association between HIV infection and overall TB cases is well characterized, but the relationship between EPTB and HIV infection is less clear. EPTB is a relatively common presentation in individuals with HIV infection, although pulmonary

disease is most often associated with HIV infection. Risk factors for EPTB are not clear. Some studies have indicated that besides HIV positivity, patients with liver cirrhosis and diabetes are also at increased risk. Genetic descent (e.g., Asia Pacific and Native American) has been proposed as a risk factor as well.

Miliary tuberculosis refers to the seeding of many organs outside the pulmonary tree with AFB through hematogenous spread. This usually occurs shortly after primary pulmonary disease but can take place at any point during acute or chronic TB. The most common sites of spread of *M. tuberculosis* are the lymph nodes, spleen, liver, lungs, bone marrow, and kidneys. Other forms of EPTB include pleuritis, lymphadenitis, meningitis, peritonitis, and gastrointestinal and skeletal infections. Almost any organ of the body can be infected by *M. tuberculosis*. However, diagnosing EPTB is challenging because clinical samples must be obtained from relatively inaccessible sites, and often only a few bacteria are present, thus decreasing the sensitivity of diagnostic tests.

Overall, children account for most cases of miliary TB, but it is also a common form of TB in individuals with HIV infection. The mortality is 20% or higher in most studies; the finding of meningitis is an extremely poor prognostic indicator. Up to 70% of patients with HIV infection may have EPTB alone or, usually, in combination with pulmonary disease. The most common extrapulmonary sites in this population are the lymph nodes (especially mediastinal), **genitourinary tract, and abdominal cavity**. Bacteremia is not uncommon.

Lymphadenitis is usually a disease of children, appearing as painless head or neck swellings. Genitourinary TB can involve the kidneys and genital organs. Renal TB accounts for 2% of all cases of TB and manifests itself as typical urinary tract symptoms and sterile pyuria. Cultures may be positive in up to 80% of cases. Male genital TB usually appears as a scrotal mass and frequently occurs along with renal TB. Skeletal TB of the spine is referred to as Pott disease. Back pain is the most common characteristic. Cultures of bone and tissue are needed to confirm the diagnosis. Peripheral skeletal bones and joints also may be involved, with the hip and knee being the most common sites.

Meningitis caused by *M. tuberculosis* is usually the result of a rupture of a tubercle into the subarachnoid space and not usually via hematogenous spread. In childhood, it occurs rarely after primary pulmonary infection. Most infections occur at the base of the brain; patients may develop very thick, gelatinous, masslike lesions there. With more chronic disease, a fibrous mass may surround cranial nerves. Involvement of arteries can cause infarctions. Cerebrospinal fluid (CSF) examination usually reveals an elevated protein level, a decreased glucose level, and a predominance of lymphocytes.

Diagnosis of *Mycobacterium tuberculosis* infection

Diagnosis of primary TB is usually limited to signs and symptoms, chest roentograph, and a positive PPD skin test or interferon-gamma release assay (IGRA) result. Cultures are confirmatory, but because of the slow-growing nature of *M. tuberculosis*, a culture result can take up to 8 weeks. Children may demonstrate a nonproductive cough and fever, with or without shortness of breath; these symptoms are unusual in adults. Chest radiography usually shows normal results, although, rarely, there may be infiltrates without cavitation in the anterior segment of the upper, middle, or lower lobe,

with hilar or paratracheal lymphadenopathy. Along with these limited clinical findings, patients with primary TB can have a paucity of bacteriologic findings. If sputum or bronchial washings are cultured during the primary infection, the positivity rate is only 25% to 30%.

The tuberculin skin test has been used for many years to determine an individual's exposure to *M. tuberculosis*. Protein extracted and purified from the cell wall of culture-grown *M. tuberculosis* is used as the antigen (i.e., PPD). A standardized amount of antigen is injected intradermally into the patient's forearm. The skin test detects a patient's cell-mediated immune (CMI) response to the bacterial antigens in a type IV hypersensitivity reaction. Reactivity is read at 48 hours. In immunocompetent individuals, the presence of a raised firm area (induration) 10 mm or larger is considered reactive. A reactive tuberculin skin test only indicates past exposure to *M. tuberculosis*. Other *Mycobacterium* spp. generally result in an induration smaller than 10 mm. Immunocompromised patients with previous *M. tuberculosis* infection may also produce induration less than 10 mm. Some countries use attenuated *Mycobacterium bovis* (BCG) as a vaccine against TB. Individuals who have received this vaccine will have a reactive PPD skin test.

Because some people with latent TB infection have a negative reaction when tested years after being infected, the **two-step PPD skin test is often recommended**. First, a routine PPD skin test is administered. If the result is reactive, no additional skin testing is needed. If the result is nonreactive, a second PPD skin test is administered in 1 to 3 weeks. If the second test is also nonreactive, the individual is considered not infected. A reactive test indicates a boosted response for an infection many years ago. The individual should be evaluated to determine if treatment is necessary.

IGRAs have several advantages over skin testing and have become more frequently used. The Quantiferon-TB Gold Plus assay (Cellestis, Carnegie, Victoria, Australia) and the T-SPOT.TB (Oxford Immunotec, Oxford, England) measure the CMI response in whole-blood samples to mycobacterial antigens. These assays have a greater specificity than the PPD skin test and are just as sensitive if not more so. IGRAs have reported sensitivities and specificities of 88% to greater than 95%. Like skin testing, however, the sensitivities of IGRAs in children and those who are immunosuppressed, such as patients with AIDS, are lower compared with adults and immunocompetent persons.

Unlike the tuberculin skin test, IGRAs are not affected by BCG vaccination and do not cross-react with antigens from most other mycobacterial species. They are also free from the bias that might be associated with reading and interpreting the tuberculin skin test results. In addition, patients need to be seen only once by a health care provider and do not need to return, as is necessary for reading a tuberculin skin test. Results are available in about 24 hours. However, these tests are more expensive than tuberculin skin testing. Both IGRA methods measure IFN- γ production by T cells that have been stimulated by two or three secretory, low-molecular-weight mycobacterial peptides: early-secreted antigenic target 6 (ESAT-6), culture filtrate protein 10 (CFP-10), or TB 7.7. Like the tuberculin skin test, IGRAs cannot distinguish between active and latent infections.

Cultures, while time-consuming, have been considered the reference method for diagnosing most *Mycobacterium* infections. Isolation of mycobacteria is covered in more detail later

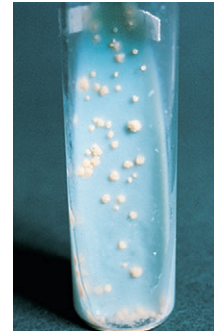


Fig. 26.1 *Mycobacterium tuberculosis* growing on Löwenstein-Jensen medium.

in this chapter. Colonies of the slowly growing *M. tuberculosis* are typically raised, with a dry, rough appearance. The colonies are nonpigmented and classically described as being buff colored (Fig. 26.1). Elaboration of cord factor can result in characteristic cord formation. Optimal growth occurs at 35° to 37° C. *M. tuberculosis* is characteristically positive for niacin accumulation, reduction of nitrate to nitrite, and production of catalase, which is destroyed after heating (heat-stable catalase negative). Isoniazid-resistant strains may not produce catalase at all. *M. tuberculosis* is inhibited by nitroimidazopyran or *p*-nitroacetylaminobenzoyl- β -propiophenone (NAP). This species can be distinguished from *M. bovis* by the inhibition of *M. bovis* by thiophene-2-carboxylic acid hydrazide (T2H) and pyrazinamidase activity.

Treatment

The treatment of TB involves the use of more than one antimycobacterial agent in two phases for 4, 6 or 9 months. Currently, 10 drugs are approved by the U.S. Food and Drug Administration (FDA) for treating TB. Typically, phase one (intensive phase) treatment of pulmonary TB disease involves 8 weeks of therapy with isoniazid, rifampin, pyrazinamide, and ethambutol. Phase two (continuation phase) treatment consists of isoniazid and rifampin for an additional 18 weeks. In most individuals, AFB are cleared from the sputum within the first 2 months.

Although people with latent TB infection are asymptomatic and cannot spread TB, the bacteria can reactivate and progress to active TB disease. Therefore not only should people with TB disease be treated but also those individuals with latent TB infection. An estimated 80% of the people in the United States who develop TB disease do so because of untreated latent TB infection. Several regimens are used to treat latent TB infection. Short-course, rifamycin-based, 3- or 4-month regimens are recommended. To insure patient compliance, the WHO and the Centers for Disease Control and Prevention (CDC) recommend directly observed therapy.

Multidrug-resistant Mycobacterium tuberculosis

Multidrug-resistant tuberculosis (MDR-TB) is defined as resistance to at least isoniazid and rifampin, drugs recognized as the primary treatments for drug-susceptible *M. tuberculosis*. The incidence of MDR-TB in the United States has remained relatively stable, around 1%, since 1996.

Within any population of *M. tuberculosis*, resistance to a single agent can develop at a fairly well-defined rate. For example, with isoniazid and streptomycin, the chance that a resistant isolate will develop is approximately 1 in 10^6 . The rate of spontaneous mutation of resistance to both drugs in one cell is the product of the rates of resistance to the individual drugs, or 1 in 10^{12} . In a patient with pulmonary TB, the pulmonary cavity may contain 10^7 to 10^9 bacterial cells. Random drug resistance has a good likelihood of developing when only one antimycobacterial agent is used or if the patient is receiving multidrug therapy and fails to complete the course of medication. Therefore combination therapy (i.e., two or more drugs) to treat mycobacterial infections is recommended.

Risk factors for drug resistance may include previous treatment for TB, residence in an area endemic for drug resistance, or close contact with an individual who has MDR-TB. Drug resistance is usually acquired by spontaneous mutations because of inappropriate use of antimicrobial agents to treat *M. tuberculosis* and lack of patient adherence. If adherence is an issue, directly observed therapy is recommended to ensure proper treatment. Otherwise, resistance may be assumed, and in vitro testing should be performed.

MDR-TB requires an extended treatment period compared with drug-susceptible isolates. Second-line anti-TB drugs may include a regimen of four or five drugs that include an aminoglycoside, fluoroquinolone, pyrazinamide, and cycloserine/terizidone, among others. With the numbers of cases of MDR-TB increasing worldwide, newer agents are being tested in vitro to determine their efficacy.

In 2016, the WHO estimated that 6.2% of MDR-TB cases were extensively drug-resistant tuberculosis (XDR-TB). The CDC defines XDR-TB as TB resulting from strains resistant to rifampin (MDR/RR-TB) and isoniazid plus any fluoroquinolone and at least one of three injectable second-line anti-TB drugs (i.e., amikacin, kanamycin, or capreomycin). In 2021, the WHO published a new definition of XDR-TB, which is "TB caused by *Mycobacterium tuberculosis* (*M. tuberculosis*) strains that fulfill the definition of MDR/RR-TB and which are also resistant to any fluoroquinolone and at least one additional Group A drug (Group A drugs are the most potent group of drugs in the ranking of second-line medicines for the treatment of drug-resistant forms of TB using longer treatment regimens and comprise levofloxacin, moxifloxacin, bedaquiline and linezolid)."

XDR-TB has been reported in all regions of the world but is most frequently found in countries of the former Soviet Union and in Asia. Because of the threat of MDR-TB and XDR-TB, it is important for laboratories to identify the *Mycobacterium* spp. rapidly and perform antimicrobial susceptibility testing so appropriate therapy can be administered as quickly as possible.

Vaccine

The BCG vaccine, an attenuated *M. bovis*, is used in many countries with endemic TB. The vaccine is more efficacious in preventing infection when administered to children compared with adults. However, the vaccine cannot be given to immunocompromised individuals, such as those with HIV infection. It is therefore unlikely to be useful in countries with a high prevalence of HIV infection. Individuals who have received the vaccine will have a positive tuberculin (PPD) skin test result.

Mycobacterium bovis

Mycobacterium bovis causes TB primarily in cattle but also in other ruminants, as well as in dogs, cats, swine, parrots, and humans. The disease in humans closely resembles that caused by *M. tuberculosis* and is treated the same way. In some areas of the world, a significant percentage of cases of TB are caused by *M. bovis*, but in the United States, the number of isolates of this organism is very low.

M. bovis is closely related taxonomically to *M. tuberculosis* and belongs to the *M. tuberculosis* complex. It grows very slowly on egg-based media, producing small, granular, rounded, nonpigmented colonies with irregular margins after 21 days of incubation at 37° C. On Middlebrook 7H10 medium, colonies resemble those of *M. tuberculosis* but are slower to mature. Most strains of *M. bovis* are niacin negative, do not reduce nitrate, and do not grow in the presence of 2 µg/mL T2H, characteristics that distinguish the species from most strains of *M. tuberculosis*.

Clinical significance of nontuberculous mycobacteria

Over 180 species of NTM are recognized, and about one half are classified as slowly growing. Most NTM are found in soil and water. They have been implicated as opportunistic pathogens in patients with underlying lung disease (e.g., cystic fibrosis, chronic obstructive pulmonary disease), immunosuppression, or percutaneous trauma. AIDS has contributed greatly to the incidence and awareness of NTM disease. Chronic pulmonary disease resembling TB is the usual clinical presentation associated with these organisms, although a few species are more often associated with cutaneous infections. Some species can be commensal inhabitants of humans; therefore their isolation must be evaluated with clinical history and presentation. NTM infections are not considered transmissible from person to person or animal to person.

Slowly growing species

Mycobacterium avium Complex

Epidemiology

Currently, 11 species belong to the *Mycobacterium avium* complex (MAC), with *Mycobacterium avium* and *Mycobacterium intracellulare* being the most common species isolated from humans. These organisms are common environmental saprophytes and have been recovered from soil, water, house dust, and other environmental sources. Certain areas, such as coastal marshes, have higher concentrations of the organism. Environmental sources, especially natural waters, seem to be the reservoir for most human infections.

M. avium is a cause of disease in poultry, cattle, and swine, but animal-to-human transmission has not been shown to be an important factor in human disease. The three subspecies found in animals are rarely associated with human infections; this may explain why animal-to-human transmission is uncommon. A fourth subspecies, *M. avium* subsp. *hominissuis*, is responsible for most human infections. MAC is the

leading causes of NTM infections in humans. A large increase in MAC infections occurred in the past primarily because of the increased number of infections in patients with AIDS. Although not a reportable disease, recently MAC infections have been decreasing among persons with HIV infection.

Clinical infections

Pulmonary disease resulting from MAC infection can have a variety of clinical presentations. The most common form is a slowly progressive, chronic cavitary disease in middle-age men with a history of smoking and other underlying pulmonary disease. The disease resembles the clinical picture of TB—cough, fatigue, weight loss, low-grade fever, and night sweats. Other presentations include lymphadenitis and tenosynovitis. Disseminated disease is common in immunocompromised patients, such as those with AIDS, or in patients with hematologic abnormalities. MAC infections are the most common systemic bacterial infection in patients with AIDS. The loss of CD4⁺ T cells reduces the activation of macrophages to kill MAC organisms. NTM pediatric lymphadenitis previously involved *M. scrofulaceum* in the 1970s; however, MAC has been associated in approximately 80% of recently reported cases.

The clinical outcome of MAC lung disease is unpredictable, so management of patients can be difficult. Observation, therapy for underlying pulmonary disease (e.g., bronchodilators, broad-spectrum antimicrobials, smoking cessation), and periodic sputum cultures may be all that is required for most patients. For patients with significant symptoms and advanced or progressive radiographic disease, multidrug therapy is indicated. For children with cervical lymphadenitis, excision surgery without chemotherapy is usually successful. A combination of surgical excision and chemotherapy is the usual treatment for adults with localized nonpulmonary disease. Most isolates of MAC are resistant to commonly used antimycobacterial drugs. However, many cases of disseminated disease in immunosuppressed patients without AIDS respond to multidrug regimens. Multidrug therapy consisting of clarithromycin or azithromycin along with ethambutol and rifampin has resulted in symptomatic reduction and clinical improvement in many, but not all, patients.

Laboratory diagnosis

Because the two most common human isolates in the MAC are so similar, most laboratories do not distinguish between them but report isolates of both species as MAC. On primary isolation media, these organisms grow slowly, producing thin, transparent or opaque, homogeneous smooth colonies. A small proportion of strains may exhibit rough colonies. Usually, the colonies are nonpigmented, but they may become yellow with age. Rarely are the colonies pigmented from the onset of detectable growth. The optimal growth temperature is 37° C.

On microscopic examination, the cells are short, coccobacillary, and uniformly stained, without beading or banding. Long, thin, beaded bacilli resembling *Nocardia* spp. may be seen in stains of very young cultures or under certain other conditions. MAC species are inactive in most physiologic tests used to identify the mycobacteria. Exceptions are the production of a heat-stable catalase and the ability to grow on media containing 2 µg/mL T2H. Nucleic acid probes are available for the identification of MAC and the two species. Nucleic acid probes for the identification of *M. avium* and *M. intracellulare* are commercially available. Phenotypic characteristics

are insufficient for species identification of the slowly growing mycobacteria. Molecular methods are now the reference method.

Mycobacterium avium subsp. *paratuberculosis*

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the causative agent of Johne disease, an intestinal infection occurring as a chronic diarrhea in cattle, sheep, goats, and other ruminants. MAP has also been reported to cause a diarrheal/wasting disease in nonhuman primates. MAP is found in potable water, pasteurized milk, and other foods. Researchers have found a significantly higher percentage of anti-MAP antibodies in individuals with Crohn disease compared with individuals without irritable bowel disease. These studies suggest a link between MAP and Crohn disease. However, a causal relationship between the presence of MAP infection and autoimmune disease has not been demonstrated. MAP is difficult to cultivate because of its very slow growth rate (3 to 4 months) and its need for mycobactin-supplemented medium for primary isolation. Mycobactin is an iron-binding compound produced by other mycobacterial species.

Mycobacterium kansasii

Epidemiology

Mycobacterium kansasii is second to MAC as the cause of NTM lung disease. Tap water seems to be the most significant reservoir. The natural source of human infection is unclear but is likely by aerosol. As with other NTM, infections are not normally considered contagious from person to person.

Clinical infections

The most common manifestation is chronic pulmonary disease, closely resembling TB, involving the upper lobes, usually with evidence of cavitation and scarring. Most patients with pulmonary disease have underlying conditions including chronic obstructive pulmonary disease, bronchiectasis, pneumoconiosis, alcohol abuse, malignancies, and HIV infection. Extrapulmonary infections, such as lymphadenitis, skin and soft tissue infections, and joint infection, have been reported occasionally. Disseminated *M. kansasii* infection rarely occurs in immunocompetent individuals but has been reported in severely immunocompromised patients, particularly those with AIDS. In fact, infections are more common in HIV-infected individuals compared with those who are HIV negative.

Laboratory diagnosis

M. kansasii is a slowly growing organism that appears as long rods with distinct cross-banding. *M. kansasii* has an optimal growth temperature of 37° C, and colonies appear smooth to rough, with characteristic wavy edges and dark centers when grown on Middlebrook 7H10 agar. Some cording can usually be seen with low-power magnification. Colonies are **photochromogenic** (Fig. 26.2); that is, they form a pigment when exposed to light but are nonpigmented in the dark. With prolonged exposure to light, most strains form dark red crystals of β -carotene on the surface of and inside the colony, producing reddish-orange colonies. **Scotochromogenic** (produce pigment in light and dark) and **nonchromogenic** strains are rarely isolated. Most strains are strongly catalase positive (>45 mm in a semi-quantitative test); strains that are low catalase producers (<45 mm) are less commonly isolated.



Fig. 26.2 *Mycobacterium kansasii* growing on Löwenstein-Jensen medium showing photoreactivity. *Left*, Before exposure to light. *Right*, After exposure to light.

Characteristics that distinguish this species are a growth rate resembling that of *M. tuberculosis* at 35° C, strong photochromogenic properties, ability to hydrolyze Tween 80 in 3 days, strong nitrate reduction, and catalase production. A nucleic acid probe for the identification of *M. kansasii* isolates is commercially available. Molecular analysis has identified five subtypes; the one most commonly found in humans is subtype 1.

Mycobacterium genavense

Mycobacterium genavense has been reported as a cause of disseminated infections in patients with AIDS. It has also been associated with enteritis and genital and soft tissue infections in HIV-positive and HIV-negative immunocompromised patients. These slow-growing, fastidious mycobacteria have been recovered in BACTEC culture systems but failed to grow on subculture to routine solid media. Growth was obtained when Middlebrook 7H11 agar was supplemented with mycobactin. Isolates yield positive test results for semi-quantitative and heat-stable catalase, pyrazinamidase, and urease.

Mycobacterium haemophilum

The rare infections associated with *Mycobacterium haemophilum* occur primarily in patients who are immunocompromised. Approximately one half of the reported cases have occurred in patients with AIDS. Infections typically present as skin nodules on the extremities. Submandibular lymphadenitis, subcutaneous nodules, painful swellings, ulcers progressing to abscesses, and draining fistulas are often seen. *M. haemophilum* also causes cervical lymphadenopathy in otherwise healthy children.

A unique characteristic of this organism is its requirement for hemoglobin or hemin for growth. Isolation of this species is accomplished on media supplying the needed growth supplement, such as chocolate (CHOC) agar, Mueller-Hinton agar with 5% Fildes enrichment, and LJ medium containing 2% ferrous ammonium citrate. Successful isolation on Middlebrook 7H10 agar with an X factor disk planted in the inoculated

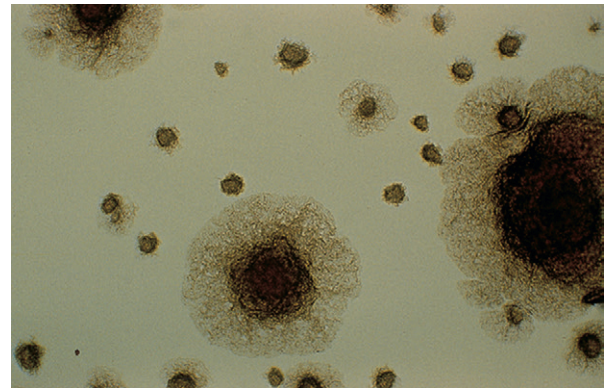


Fig. 26.3 *Mycobacterium marinum* on Middlebrook 7H10 medium producing rough colonies (×20).

area has been reported. Optimal growth temperature is 28° to 32° C; little or no growth occurs at 37° C. Colonies are rough to smooth and nonpigmented. Microscopically, the cells are strongly acid-fast, short, occasionally curved bacilli without banding or beading, and arranged in tight clusters or cords.

Mycobacterium marinum

Mycobacterium marinum has been implicated in diseases of fishes and is isolated from fishes in aquariums. Cutaneous infections in humans occur when salt water, or inadequately chlorinated freshwater containing the organism contacts traumatized skin. Outbreaks of cutaneous lesions in lifeguards have been reported. The typical presentation is a tender red or blue-red subcutaneous nodule, or “swimming pool granuloma,” usually occurring on the elbow, knee, toe, or finger 2 to 3 weeks after exposure. In some cases, an ulcer develops at the primary site of inoculation, with secondary spread along the ascending lymphatics. Treatment modalities include simple observation of minor lesions, surgical excision, anti-TB drug therapy, and the use of single antimicrobial agents. In vitro susceptibility testing shows that *M. marinum* is susceptible to rifampin and ethambutol, resistant to isoniazid and PZA, and partially resistant or intermediate to streptomycin.

M. marinum cells are moderately long to long rods with cross-barring. Colonies of this slowly growing organism are smooth to rough (Fig. 26.3) and wrinkled on inspissated egg medium but may be smooth when grown on Middlebrook 7H10 or 7H11 agar. *M. marinum* is photochromogenic; young colonies grown in the dark may be nonpigmented or buff, whereas colonies exposed to light develop a deep yellow color. Growth is optimal at incubation temperatures of 28° to 30° C. Preferences for growth at lower incubation temperatures, along with photochromogenicity, are clues to the identification of *M. marinum*. Some strains of *M. marinum* produce niacin; however, none reduce nitrate or produce heat-stable catalase. The organisms hydrolyze Tween 80 and produce urease and pyrazinamidase.

Mycobacterium scrofulaceum

Although rare in the United States, this bacterium can cause pulmonary tract infections in adults, and before being replaced by the MAC, *M. scrofulaceum* was the most common cause of cervical lymphadenitis in children. The infection manifests itself in one or more enlarged nodes, often adjacent



Fig. 26.4 *Mycobacterium scrofulaceum* on Löwenstein-Jensen medium.

to the mandible and high in the neck, with little or no pain. Patients are usually treated by surgical incision and drainage; anti-TB drugs are generally not necessary. Pulmonary infections caused by *M. scrofulaceum* typically appear with chronic structural lung disease or an immunomodulatory condition. *M. scrofulaceum* is often resistant to isoniazid, streptomycin, ethambutol, and *p*-aminosalicylic acid.

M. scrofulaceum is a uniformly staining, acid-fast, medium to long rod. The organism grows slowly (4 to 6 weeks) at incubation temperatures ranging from 25° to 37° C. Colonies are smooth with dense centers and pigmentation from light yellow to deep orange. The organism is scotochromogenic (Fig. 26.4). Members of this species do not hydrolyze Tween 80 or reduce nitrate but do produce urease and are high (>45 mm) catalase producers. These characteristics aid in differentiating this organism from other slowly growing scotochromogens, including certain strains of MAC, *M. gordonae*, and *M. szulgai*.

Mycobacterium simiae

The original strains of *Mycobacterium simiae* were isolated from the lymph nodes of monkeys. Although the organism has been recovered from tap water, there seems to be significant geographic variation in the incidence. For example, *M. simiae* is rarely isolated in most parts of the United States, but in parts of Texas, it is a relatively common isolate. Infrequent cases of human infection from *M. simiae* have been reported and are often associated with HIV-positive patients. Infection typically manifests as pulmonary disease, but lymphadenitis, skin lesions, and other presentations have been reported. Many isolates are resistant to most anti-TB drugs and are associated with significant morbidity and mortality.

Cells of *M. simiae* appear as short coccobacilli. When they are grown on inspissated egg medium at 37° C, smooth colonies appear in 10 to 21 days. Colonies on Middlebrook 7H10

agar are thin, transparent, tiny, and filamentous. The species is usually photochromogenic. Development of the yellow pigment may require prolonged incubation, whereas some strains may fail to produce pigment on exposure to light. Differential biochemical characteristics are the accumulation of niacin, negative nitrate reduction, and high-level, heat-stable catalase. *M. simiae* is one of the very few NTM that produce niacin, making it possible to confuse the identification with *M. tuberculosis* if pigment production under light is not observed.

Mycobacterium szulgai

A rare clinical isolate, the most common manifestation of *Mycobacterium szulgai* is pulmonary disease resembling TB. Extrapulmonary infections, including tenosynovitis of the hand, bursitis, osteomyelitis, keratitis, cervical lymphadenitis, and renal or cutaneous infection, have also been reported. This organism has been isolated from environmental sources such as snails, tropical fish, aquarium water, swimming pools, and hospital water supplies. Person-to-person transmission has not been documented. It is susceptible in vitro to most anti-TB drugs, and combination treatment is generally successful.

M. szulgai cells are medium to long rods, with some cross-barring. When cultured on egg-based medium at 37° C, the organism produces smooth and rough colonies. At 37° C, yellow-to-orange pigment develops in the absence of light and intensifies with exposure to light. Colonies grown at 22° C are nonpigmented or buff in the absence of light and develop yellow-to-orange pigment with light exposure. Characteristics that differentiate *M. szulgai* from other slowly growing mycobacteria are slow hydrolysis of Tween 80, positive nitrate reduction, and inability to grow in the presence of 5% sodium chloride.

Mycobacterium ulcerans

Mycobacterium ulcerans is a rare cause of mycobacteriosis in the United States but may be underreported because of difficulty in isolation. Worldwide, *M. ulcerans* is the third most common *Mycobacterium* spp., behind *M. tuberculosis* complex and *M. leprae*. The disease manifests as a painless nodule under the skin following trauma. A shallow ulcer, also referred to as a *Buruli ulcer*, develops that can be quite severe. Widespread ulcerative necrosis, osteomyelitis, and disfigurement can result. Patients rarely develop fever or systemic symptoms.

M. ulcerans cells are moderately long, without beading or cross-banding. These bacteria are fastidious and difficult to grow in vitro. Optimal growth temperature is 28° to 30° C, with little growth at 25° C and usually none at 37° C. The organism grows slowly, often requiring 6 to 12 weeks of incubation before colonies are evident. Colonies are smooth or rough and nonpigmented or lightly buff, and they do not develop pigment with exposure to light. *M. ulcerans* produces a heat-stable catalase but is inert in most other conventional biochemical tests. Molecular techniques are available that may improve detection.

Mycobacterium xenopi

Mycobacterium xenopi has been recovered from hot- and cold-water taps (including water storage tanks of hospitals)

and birds. The organism was first isolated from an African toad (*Xenopus laevis*) and was considered nonpathogenic for humans until 1965. Isolation of *M. xenopi* is relatively uncommon in the United States. Human cases of *M. xenopi* infection are mostly slowly progressive pulmonary infections in individuals with a predisposing condition, such as chronic obstructive pulmonary disease. The pulmonary infections present a clinical picture resembling that seen in patients with *M. tuberculosis*, *M. kansasii*, or MAC infection. Disseminated and extrapulmonary infections have also been reported, primarily in patients who are immunocompromised. Strains of *M. xenopi* are susceptible to the quinolones (ciprofloxacin, ofloxacin); some isolates are susceptible to vancomycin, erythromycin, or cefuroxime. In vitro susceptibility to anti-TB drugs is variable, with resistance only to ethambutol being the most common pattern.

M. xenopi cells are long filamentous rods. Colonies of this slowly growing mycobacterium on Middlebrook 7H10 agar are small, with dense centers and filamentous edges. Microscopic magnification observation of colonies growing on cornmeal-glycerol agar reveals distinctive round colonies with branching and filamentous extensions; aerial hyphae-like elements are usually seen in rough colonies. Furthermore, young colonies grown on cornmeal agar have a "bird's nest" appearance, with characteristic sticklike projections. Optimal growth temperature is 45° C; the organism grows more rapidly at this temperature than at 37° C and fails to grow at 25° C. *M. xenopi* has been classified with the nonphotochromogenic group; however, colonies are frequently bright yellow on primary isolation when incubated in the absence of light and when exposed to light. Distinctive characteristics, in addition to optimal growth at 42° C and yellow scotochromogenic pigment, are negative reactions for niacin accumulation, nitrate reduction, and positive reactions for heat-stable catalase, arylsulfatase, and pyrazinamidase.

Rapidly growing species

The three most important rapidly growing mycobacteria causing human infections are *Mycobacterium abscessus* subsp. *abscessus* (formerly *M. abscessus*), *Mycobacterium chelonae*, and the *Mycobacterium fortuitum* group. The *M. chelonae*/*M. abscessus* complex makes up one group within the rapidly growing mycobacteria. Posttraumatic wound infections are the most common infection associated with the rapidly growing mycobacteria. The *M. fortuitum* group causes about 60% of localized cutaneous infections.

Mycobacterium chelonae/*Mycobacterium abscessus* group

M. chelonae is found in the environment and is associated with many of the same opportunistic infections as those associated with the *M. fortuitum* group. *M. chelonae* is related closely to *M. abscessus* subsp. *abscessus* and is a commonly isolated rapidly growing *Mycobacterium*. It has caused sporadic cases of localized wound infections following medical or surgical procedures, including needle injections. The *M. chelonae*/*M. abscessus* complex is the most likely among the rapidly growing mycobacteria to be isolated from disseminated cutaneous infections in immunocompromised patients. *M. chelonae* has been associated with a variety of

infections of the skin, lungs, bone, central nervous system, and prosthetic heart valves. Approximately 80% of the cases of pulmonary disease resulting from rapidly growing mycobacteria are caused by *M. abscessus*. Infections by this organism have also been seen in patients with cystic fibrosis. Unlike with *M. chelonae*, tap water is an important reservoir for *M. abscessus*.

Microscopically, young cultures of *M. chelonae* are strongly acid fast, with pleomorphism ranging from long and tapered to short and thick rods. This rapidly growing *Mycobacterium* produces rough or smooth, nonpigmented to buff colonies within 3 to 5 days of incubation at 28° to 30° C. A positive 3-day arylsulfatase test result, nitrate reduction negative, and growth on MacConkey agar without crystal violet are characteristics that help differentiate *M. chelonae* and *M. abscessus* from other nonchromogenic, rapidly growing mycobacteria.

Mycobacterium fortuitum group

The *M. fortuitum* group contains 10 species. Common in the environment, *M. fortuitum* has been isolated from water, soil, and dust. The *M. fortuitum* group is the most common rapidly growing *Mycobacterium* associated with localized cutaneous infections, bone and joint infections, and abscesses at the site of puncture wounds. Infections associated with long-term use of intravenous and peritoneal catheters, injection sites, and surgical wounds following mammoplasty and cardiac bypass procedures have also been reported. Differences in susceptibility to antimicrobial agents occur among the rapidly growing species; thus in vitro susceptibility testing is often recommended for clinically significant isolates.

After 3 to 5 days of incubation at 35° C, *M. fortuitum* colonies appear rough or smooth and nonpigmented, creamy white, or buff. Microscopic examination of growth on cornmeal-glycerol and Middlebrook 7H11 agars after 1 to 2 days of incubation reveals colonies with branching filamentous extensions and rough colonies with short aerial elements. Cells are pleomorphic, ranging from long and tapered to short and thick rods. From most cultures, especially older ones, cells tend to decolorize and appear partially acid fast with any of the acid-fast staining techniques. Additional characteristics that distinguish *M. fortuitum* from other rapidly growing mycobacteria are the positive 3-day arylsulfatase test result and reduction of nitrate.

Mycobacterium smegmatis group

The *Mycobacterium smegmatis* group contains two species: *M. smegmatis* and *Mycobacterium goodii*. Commonly considered saprophytic, *M. smegmatis* has been implicated in rare cases of pulmonary, skin, soft tissue, and bone infections. It has been reported to cause granulomas in the soft tissue of the web space of a previously healthy 67-year-old patient who had no recollection of trauma to the hand.

Upon acid-fast staining, cells appear long and tapered or as short rods with irregular acid fastness. Occasionally, rods are curved with branching or Y-shaped forms; swollen, with deeper staining, beaded, or ovoid forms are sometimes seen. Colonies appearing on egg medium after 2 to 4 days are usually rough, wrinkled, or coarsely folded; smooth, glistening,

butyrous colonies may also be seen. Colonies on Middlebrook 7H10 agar are heaped and smooth or rough with dense centers. Pigmentation is rare or late; colonies appear nonpigmented, creamy white, or buff-to-pink in older cultures. In addition to the rapid growth rate and nonpigmented rough colonies, characteristics valuable in the identification of this organism are its negative arylsulfatase reaction, positive iron uptake, ability to reduce nitrate, and growth in the presence of 5% sodium chloride (NaCl) and on MacConkey agar without crystal violet.

Mycobacterium leprae

Mycobacterium leprae is the causative agent of Hansen disease (leprosy), an infection of skin, mucous membranes, and peripheral nerves. The disease is rare in the United States and other Western countries, yet it remains a major problem in other parts of the world. At one time, the WHO estimated that 11 million people had Hansen disease. However, the WHO launched an eradication program, and since 1985 the incidence of Hansen disease has been reduced by about 90%. Since 1995, the WHO has provided treatment free of charge to all patients with Hansen disease. Globally, the annual incidence has steadily declined since 2001 to 127,558 new leprosy cases detected in 2020. Currently, India, Brazil, and Indonesia account for about 80% of all new cases; each reported over 10,000 cases. In the United States, roughly 100 cases are reported annually, which are often acquired abroad.

Despite its reputation, Hansen disease is not highly contagious. The most important mode of transmission is not known, but the disease can be transmitted by direct contact and inhalation of aerosols from skin lesions. Shedding from the nasal passage is another route of transmission. The bacteria multiply slowly, producing a lengthy incubation period up to 20 years. The two extreme forms of the disease are tuberculoid leprosy, also called *paucibacillary Hansen disease*, and lepromatous leprosy, also called *multibacillary Hansen disease*. Symptoms of tuberculoid leprosy include hypopigmented or hyperpigmented skin macules and nerve involvement that can produce areas with loss of sensation (anesthesia). Patients eventually exhibit an effective CMI response. The optimal growth temperature for *M. leprae* is approximately 30° C, and because the patient mounts an adequate CMI response, the bacteria tend to remain in the extremities. Spontaneous recovery often occurs with tuberculoid leprosy.

Conversely, patients with lepromatous leprosy have a strong humoral-mediated immune response but not an effective CMI response. The disease is slowly progressive, and if untreated, it can be life-threatening. It is characterized by disfiguring skin lesions and progressive, symmetric nerve damage. Lesions of the mucous membranes of the nose can lead to destruction of the cartilaginous septum, resulting in nasal and facial deformities. The current recommended therapy for lepromatous leprosy consists of a combination of diaminodiphenylsulfone (dapsone) and rifampin for a minimum of 6 months. For tuberculoid leprosy, clofazimine is added, and treatment should be continued for 12 months.

The third and most common form is known as *borderline* or *dimorphous Hansen disease*. It presents as intermediate in severity. Skin lesions resemble those found in the tuberculoid form,

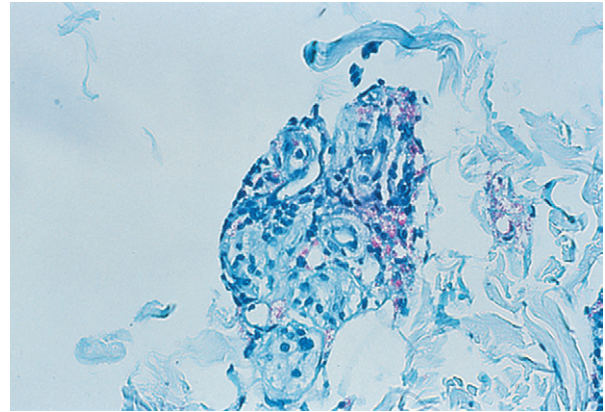


Fig. 26.5 *Mycobacterium leprae* from a skin biopsy in a patient with lepromatous leprosy (acid-fast smear stained with Ziehl-Neelsen stain, $\times 1000$).

but they are more numerous and can be found anywhere on the body. Peripheral nerves are affected, resulting in anesthesia and weakness. The lepromin skin test utilizes a standardized suspension of inactivated *M. leprae*. Like the PPD test for TB, a small volume is injected just under the skin and examined at 48 hours. Although it is not recommended for primary diagnosis, it can help differentiate between tuberculoid and lepromatous leprosy. Uninfected patients and those with lepromatous leprosy will be nonreactive, indicating a weak or negative CMI response. Individuals who were previously exposed and have a CMI response will have a reactive response, suggesting tuberculoid leprosy.

Laboratory diagnosis of Hansen disease depends on the microscopic demonstration of AFB from skin biopsy specimens. *M. leprae* has not been grown on artificial media. In patients with tuberculoid leprosy, organisms are extremely rare and may not be detected in skin scrapings or biopsy specimens. However, AFB are usually abundant in samples from patients with lepromatous leprosy (Fig. 26.5). *M. leprae* is not as acid fast or alcohol fast as in the case of other mycobacteria; as such, a weaker decolorizer consisting of 10% sulfuric acid is recommended instead of the standard acid-ethanol decolorizer. The bacteria are rod shaped, usually 1 to 7 μm long and 0.3 to 0.5 μm wide. The entire smear should be examined under oil immersion field ($\times 1000$) for the presence of the microorganisms. PCR assays are also available to definitively detect the bacteria.

Isolation and identification of mycobacteria

Growth rate, colony morphology, pigmentation, nutritional requirements, optimal incubation temperature, and biochemical test results are traditional criteria used to differentiate species within the genus *Mycobacterium* (Table 26.1). Rapid techniques include broth-based culture systems, including some that monitor cultures continuously during the incubation period. However, because phenotypic identification of the slowly growing mycobacteria is not sufficient for accurate species identification, molecular-based assays are the reference method. A limited number of species-specific nucleic acid probes offer rapid identification of culture isolates.

Table 26.1 Identification of clinically important mycobacteria^a

Descriptive term	Species (subspecies)	Colony Morphology	Temp. Growth Range (°C)	Growth Rate	Pigment	Arylsulfatase		Carbon Sources			Catalase			Growth on MacConkey Agar	Niacin	Nitrate Reduction	Pyrazinamide	Growth on 5% NaCl	Tellurite Reduction	Growth on T2H	Tween Hydrolysis + 10 Days	Tween Opacity (1 Week)	Urease ^c
						3 Days	2 Weeks	Sodium Citrate	Inositol	Mannitol	Semi-quantitative >45	Heat Stable (68° C)	Iron Uptake										
Rapid growers	<i>M. fortuitum</i>	Smooth 87%, rough 13%	22–40	Rapid	N = 99%	+ (97)	+ (99)	– (99)	– (99)	– (99)	98	+ (94)	+ (99)	+ (96)	– (99)	+ (99)		+ (75)	+ (97)	+ (99)	+ – (50)	+ (91)	+ (93)
	<i>M. peregrinum</i>	Smooth	22–37					– (99)	– (99)	+ (99)													
	<i>M. fortuitum</i> ^{d,e}	Smooth	22–37					– (99)	+ (99)	+ (99)													
	former third biovariant complex																						
	<i>M. chelonae</i>	Smooth 60%, rough 40%	22–35			+ (96)	+ (98)	+ (99)	– (99)	– (99)	(97)	+ / – (53/47)	– (98) + – (99)	+ (96)	– (92)	– (99)		– (99)	+ (85)	+ (98)	+ / – (93/47)		+ (99)
	<i>M. abscessus</i> subsp. <i>abscessus</i>		22–40					– (80)	– (99)	– (99)													
TB complex	<i>M. fallax</i> ^f	Rough	30–37	Rapid (30° C)	N = 99%	– (99)		– (99)	– (99)	– (99)		– (74)	– (99)	– (99)	– (99)	+ (99)		– (99)		+ (99)	+ (99)		+ (96)
	Other rapid growers	Smooth or rough	17–52	Slow (37° C)	P = 4% S = 38% N = 58%	– (72)	+ (75)	– (70)	+ (59)	+ (60)	+ (66)	+ (63)	– (56)	+ (52)	– (95)	– (51)		– (52)	+ (87)	+ (99)	+ (75)	V	+ (66)
	<i>M. tuberculosis</i>	Rough, cords	33–39	Slow	N = 99%	– (99)	– (93)				– (99)	– (99)		– (99)	+ (98)	+ (99)	+ (98)	– (99)	– (70)	+ (92)	+ (68)	– (83)	+ (98)
	<i>M. africanum</i>	Rough	35–38	Slow	N	– (99)	–				–	– (99)		– (99)	–	–	–	– (99)	– (99)	V	–		+ (99)
	<i>M. bovis</i>	7H10 = Rough LJ = Smooth	35–38	Slow	N = 99%	– (99)	– (87)					– (97)	– (92)		– (99)	– (95)	– (94)	– (98)	– (99)	– (55)	– (94)	– (84)	– (75)

Table 26.1 Identification of clinically important mycobacteria^a—Cont'd

Descriptive term	Species (subspecies)	Colony Morphology	Temp. Growth Range (°C)	Growth Rate	Pigment	Arylsulfatase		Carbon Sources			Catalase			Growth on MacConkey Agar	Niacin	Nitrate Reduction	Pyrazinamidase	Growth on 5% NaCl	Tellurite Reduction	Growth on T2H	Tween Hydrolysis + 10 Days	Tween Opacity (1 Week)	Urease ^c	
						3 Days	2 Weeks	Sodium Citrate	Inositol	Mannitol	Semi-quantitative >45	Heat Stable (68° C)	Iron Uptake											
Nonphotochromogens	<i>M. avium</i> complex	Smooth, rough	22–45	Slow 99%	N = 85% S = 12%	–(99)	+(51)				–(99)	+(76)		–(68)	–(99)	–(92)	+(86) 4 days	+(99) 7 days	–(99)	+(82)	+(99)	–(98)	–(99)	+(99)
	<i>M. ulcerans</i>	Smooth, rough	25–33	Slow	N	–					–	+			–	–		–		+	–		–	
	<i>M. xenopi</i>	Trans. 99%, smooth	35–45	Slow	P = 3% N = 80% S = 20%	+(76)	+(99)				+(99)	+(83)		–(99)	–(99)	–(94)	+(51)		–(99)	–(59)	+(99)	–(99)		–(99)
	<i>M. gastri</i>	Smooth	25–40	Slow 80% Rapid 20%	N = 99%	–(96)	+(99)				–(99)	–(99)		–(84)	–(99)	–(99)	+(99) 4 days	+(63) 7 days	–(99)	–(74)	+(99)	+(99)	–(99)	+(79)
	<i>M. terrae</i> complex	Smooth, few rough	22–37	Slow 77% Rapid 23%	N = 95% S = 4% P = 1%	–(99)	–(51)				+(99)	+(96)	–(99)	–(77)	–(99)	+(72)	+(63) 4 days	+(86) 7 days	–(94)	–(75)	+(99)	+(99)	–(93)	–(91)
	<i>M. triviale</i>	Rough	22–37	Slow	N = 99%	–(66)	+(99)				+(93)	+(99)		–(99)	–(97)	+(99)	+(60) 4 days	+(90) 7 days	+(99)	–(99)	+(99)	+(99)		–(86)
	<i>M. malmoense</i>	Smooth	22–37	Slow	N = 90% S = 10%	–(99)	–(89)				–(99)	+(69)			–(99)	–(99)	+		–(99)	+(71)	+(99)	+(97)		–(67)
	<i>M. haemophilum</i> (needs hemin)	Rough	22–35	Slow	N	–(99)					–	–	–		–	–	+		–	–		–		
Photochromes	<i>M. simiae</i>	Smooth	22–37	Slow	P = 91% N = 9% S = <1% P = <1%	–	–(84)				+(99)	+(95)	–(99)		+(85)	–(85)	+		–(99)	+(85)	+(99)	–(85)		+(64)
	<i>M. kansasii</i>	Smooth, rough	25–40	Slow 98% Rapid 2% Slow 39%	N = 99% S = <1% P = 99%	–(99)	+(55)				+(99)	+(95)		–(99)	–(99)	+(99)	–	–	–(99)	–(84)	+(99)	+(99)	–(64)	+(97)
	<i>M. marinum</i>	Smooth	25–35	Rapid 61% Slow 39%	P = 99% S = 97% P = 1%	–(66)	+(99)				–(79)	–(85)	–(99)	–(99)	–(80)	–(96)	+(99)		–(97)	–(79)	+(99)	+(99)		+(99)
	<i>M. asiaticum</i>	Smooth	33–37	Slow	P = 99% S = 97% P = 1%	–(99)	–(67)				+(91)	+(91)			–(99)	–(99)			–(99)	–(70)	+(99)	+(91)		–(99)
Scotochromogens	<i>S. scrofulaceum</i>	Smooth	22–37	Slow	S = 99%, P = 1%	–(99)	–(64)				+(93)	+(96)		–(99)	–(99)	–(85)	–(60) 4 days	+(99) 7 days	–(99)	–(57)	+(99)	–(98)	–(99)	+(99)
	<i>S. szulgai</i>	Rough, Smooth	22–37	Slow 99%	S = 99%, P = 1%	–(99)	–(62)				+(99)	+(81)			–(99)	+(99)	+		–(99)	+(56)	+(99)	±(50)	–(99)	+(94)
	<i>M. flavescens</i>	Smooth	25–42	Slow 41%	S = 99%	–(84)	+(78)				+(85)	+(99)		–(99)	–(99)	+(96)	+(99)		+(71)	–(67)	+(99)	+(97)	+(99)	+(71)

^aTest reactions listed as "+" or "–" followed by the percentage of strains reacting, as indicated. If no percentage is given or the space is blank, insufficient data were available or the test is of no apparent value.

^bPyrazinamidase data (Wayne method) from both Wayne and Hawkins. Unless indicated, results are those at 4 days. Strains of *M. tuberculosis* resistant to pyrazinamide are often pyrazinamidase negative. Within the *M. terrae* complex, *M. nonchromogenicum* isolates are usually "+" and *M. terrae* isolates are usually "–."

^cUrease data (Murphy-Hawkins disk method) primarily from Dr. Jean E. Hawkins, Reference Laboratory for Tuberculosis and Other Mycobacterial Diseases, Veterans Administration Medical Center, West Haven, CT, and Department of Laboratory Medicine, Yale University School of Medicine, New Haven, CT.

^dSeveral strains in this group have differed in carbon sources, as follows: "+" sodium citrate; "+" mannitol; "–" inositol.

^eThe third biovariant of the *M. fortuitum* group includes *M. septicum*, *M. porcinum*, *M. houstonense*, *M. boenickei*, *M. brisbanense*, *M. neworleanense*.

Modified from Kent, P. T. & Kubica, G. P. (1985). *Public health mycobacteriology: a guide for the level III laboratory*. Atlanta, GA: Centers for Disease Control and Prevention.

Data on *M. fallax* from Levy-Frebault, V., et al. (1983). *Mycobacterium fallax* sp. nov. *International Journal of Systematic and Evolutionary Microbiology*, 33, 336.

N, Nonphotochromogenic; P, photochromogenic; S, scotochromogenic; TB, tuberculosis; T2H, thiophene-2-carboxylic acid hydrazide; V, variable.w

Researchers have developed PCR assays that have increased the sensitivity of nucleic acid probes. Other techniques, such as high-pressure liquid chromatography (HPLC) and MALDI-TOF mass spectrometry, have been used recently to distinguish mycobacterial species and have become routine in many laboratories.

Laboratory safety considerations

The serious nature of TB and the usual airborne route of infection require that special safety precautions be used by anyone handling mycobacterial specimens. The incidence of TB (i.e., skin test positivity) in those who work in the mycobacteriology laboratory is at least three times higher than that among other laboratory personnel in a given institution. However, the hazard of working in a mycobacteriology laboratory is minimal if the laboratory is well designed, has appropriate equipment, and precautions are followed closely.

Personnel safety

The administration of the microbiology laboratory must ensure that each employee is (1) provided with adequate safety equipment, (2) trained in safe laboratory procedures, (3) informed of the hazards associated with the procedures, (4) prepared for action following an accident, and (5) monitored regularly by medical personnel. Laboratory personnel must use appropriate safety equipment and follow established protocols. A skin test (Mantoux) with PPD should be administered on the first day of employment and thereafter regularly to persons previously skin test negative. Individuals known to be previously skin test positive should be counseled regularly and referred for medical evaluation if their health status changes.

Personal protective equipment (PPE) provides safety for staff working in the mycobacteriology laboratory. All manipulations with cultures or specimens should be done with gloves and laboratory coats or gowns. In addition, respirators must be used when performing procedures outside the biological safety cabinet when aerosolization could result. The minimum level of respiratory protection is a respirator that contains a National Institute for Occupational Safety and Health–certified N series filter with a 95% efficiency rating (N-95). In addition to being trained to use the respirator, laboratory staff must be fit-tested.

Ventilation

Laboratory design and ventilation play important roles in mycobacteriology laboratory safety. Ideally, the mycobacteriology laboratory should be separate from the remainder of the laboratory and have a nonrecirculating ventilation system. The area in which specimens and cultures are processed should have negative air pressure in relation to other areas; that is, the air flow should be from clean areas (e.g., corridors) into less clean areas (e.g., the mycobacteriology laboratory). There must be 6 to 12 room air changes per hour to effectively remove 99% or more of airborne particles within 30 to 45 minutes. A much higher number of room air changes per hour can cause problems of air turbulence within the biological safety cabinets.

Biological safety cabinet

Because the route of infection by mycobacteria is primarily through inhalation, it is essential that the dispersal of organisms into the air be minimized and that inhalation of airborne bacilli be avoided. The biological safety cabinet is the single most important piece of equipment in a mycobacteriology laboratory. Various types of safety cabinets are available, including class I negative-pressure cabinets and class II vertical, laminar flow cabinets (see [Chapter 4](#)). Proper installation, maintenance, and testing are essential to their performance. Each safety cabinet should be tested and recertified at least yearly by trained personnel with special monitoring equipment. Processing clinical specimens or transferring viable cultures outside a safety cabinet should not be permitted.

To prevent the dispersal of infectious aerosols into the laboratory area, all potentially infectious materials should be tightly covered when they are outside the biological safety cabinet. Specimens should be centrifuged in aerosol-free safety carriers, and the tubes should be removed from the safety carriers only inside the biological safety cabinet. Specimens taken out of the safety cabinet for transport to a decontamination area must be covered. After the safety cabinet work area has been cleaned with disinfectant, the ultraviolet (UV) light, which kills microorganisms, inside the cabinet should be used to eliminate any further contamination of surfaces and airborne bacteria. Because of the hazards to skin and eyes associated with excess UV light, the UV light should be turned on only when the safety cabinet is not in use.

For sterilizing a wire-inoculating loop, an electric incinerator should be used within the biological safety cabinet. Single-use, disposable sterile applicator sticks or plastic transfer loops are also recommended. Splashproof discard containers must be used to prevent aerosol formation and possible cross-contamination of samples.

Use of proper disinfectant

Covering the work surface with a towel or absorbent pad soaked in a disinfectant reduces the accidental creation of infectious aerosols. In the selection of a disinfectant for the mycobacteriology laboratory, the product brochure should be consulted to ensure that the disinfectant is bactericidal for mycobacteria (tuberculocidal). Sodium hypochlorite is effective at a concentration of 0.05% to 0.5% (e.g., a 1:50 to 1:10 dilution of most household bleaches). The solution should be made fresh daily, and contact time should be 10 to 30 minutes. Sodium hypochlorite loses effectiveness in the presence of a large amount of protein material. Phenol-soap mixtures containing ortho-phenylphenol, such as Amphyl (Reckitt Benckiser North America, Wayne, NJ), or other phenolic derivatives are effective with contact periods of 10 to 30 minutes. Solutions of 5% phenol are no longer recommended because of toxicity to humans.

Specimen collection

Mycobacteria can be recovered from a variety of clinical specimens, primarily those from the lower respiratory area but also urine, feces, blood, CSF, tissue biopsy material, and aspirates of any tissue or organ. Thus successful isolation of mycobacteria from clinical specimens begins with properly collected and handled specimens. Whenever possible,

diagnostic specimens should be collected before the initiation of therapy. All specimens should be transported to the laboratory immediately after collection. If immediate transport is not possible, the specimen may be refrigerated overnight. Ideally, laboratories should process specimens for mycobacteria daily because delays in processing may lead to false-negative cultures and increased bacterial contamination.

Each specimen should be confined to a single collection in an individual collection container recommended by the laboratory providing the requested diagnostic service. The most recommended container is a sterile, wide-mouthed cup with a tightly fitted lid. Special sterile receptacles containing a 50-mL centrifuge tube for sputum collection are also available commercially. Because of small sample volumes, the use of swabs for clinical specimens is discouraged. As with all specimens for microbiological examination, aseptic collection is important.

The spectrum of illness caused by *Mycobacterium* spp. is so broad that almost any site can yield an acceptable specimen. Each specimen type, even when properly collected, transported, and processed, may have an intrinsic maximal yield. This can be the result of tubercle burden at the collection site or of environmental effects, such as pH, that can affect recovery. Emphasis should be placed on collecting the number and types of specimens that when transported and processed correctly maximize the diagnostic yield. Box 26.2 lists the types of clinical samples acceptable for mycobacteriologic culture.

Sputum and other respiratory secretions

Although a variety of clinical specimens may be submitted to the laboratory to recover *M. tuberculosis* and NTM, respiratory secretions, such as sputum and bronchial aspirates,

are the most common. An early-morning specimen should be collected on 3 consecutive days, although studies have suggested that the addition of a third specimen does not significantly increase the sensitivity of detecting mycobacteria. Pooled specimens are unacceptable because of increased contamination with oral microbiota. The number of specimens necessary to obtain culture confirmation and perform susceptibility testing is related to the frequency of smear positivity. If at least two of the first three sputum direct smears are positive, then three specimens are often sufficient to confirm a diagnosis. However, when none or only one of the first three sputum smears is positive, additional specimens are needed for culture confirmation. Smear positivity and culture yield differ with the extent of the disease (i.e., whether there is cavitary or noncavitary pulmonary disease or endobronchial or laryngeal disease).

A volume of 5 to 10 mL of sputum produced by deep coughing and expectoration of sputum or induced by inhalation of an aerosol of hypertonic saline should be used. Induced sputum specimens increase the chances of detection and yield of mycobacteria. When sputum is not obtainable, bronchoscopy may be performed to obtain samples, such as bronchial washing, bronchoalveolar lavage (BAL), or transbronchial biopsy specimen. Brushings appear to be more commonly diagnostic compared with washing or biopsy specimens, possibly because of an inhibitory effect on the mycobacteria caused by the lidocaine used during bronchoscopy or because of dilution of the specimen with saline. Often patients can produce sputum for several days after bronchoscopy; these samples should be collected and examined.

Gastric aspirates and washings

Gastric aspirates are used to recover mycobacteria that may have been swallowed during the night. Use of this type of specimen should be only for patients who do not produce sputum by aerosol induction, for children younger than 12 years of age, and for nonambulatory individuals. Because children have difficulty producing sputum, gastric aspiration is recommended. Gastric lavage has been reported to be better than BAL for the detection of mycobacteria in children. When BAL was compared with three morning gastric lavage specimens, diagnosis of childhood pulmonary TB could be made in 10% with BAL versus 50% with morning gastric lavage. This has not appeared to be the case in adults. Gastric lavage may offer a diagnostic alternative only for those unable to expectorate sputum and in whom BAL might be contraindicated. Gastric aspirates should be obtained in the morning after overnight fasting. Three specimens should be collected within 3 days. Sterile water, 30 to 60 mL, is instilled orally or via nasogastric tube aspiration. Prolonged exposure to gastric acid kills mycobacteria and diminishes culture yield. Specimen processing should be done expeditiously, or the specimen should be neutralized with sodium carbonate or another buffer to pH 7.0 as soon as possible after specimen collection.

Urine

For examination of urine, first-morning midstream specimens collected on 3 successive days are preferred. The entire volume of voided urine, or a minimum of 15 mL, is collected

BOX 26.2 Acceptable specimens for mycobacterologic culture

Respiratory specimens

- Spontaneously expectorated sputum
- Normal saline-nebulized, induced sputum
- Transtracheal aspirate
- Bronchoalveolar lavage
- Bronchoalveolar brushing
- Laryngeal swab
- Nasopharyngeal swab

Body fluids

- Pleural fluid
- Pericardial fluid
- Joint aspirate
- Gastric aspirate
- Peritoneal fluid
- Cerebrospinal fluid
- Stool
- Urine
- Pus

Body tissues

- Blood
- Bone marrow biopsy/aspirate
- Solid organ
- Lymph node
- Bone
- Skin

in a sterile container. A specimen may be collected through an indwelling catheter with a sterile needle and syringe. Urine specimens should be refrigerated during the interval between collection and processing; specimens should be processed promptly. As a rule, pooled specimens collected over 12 to 24 hours are not recommended. Such specimens are more subject to contamination and may contain fewer viable tubercle bacilli.

Stool

Examination of stool specimens for the presence of AFB can be useful in identifying patients, such as those with AIDS, who may be at risk for developing disseminated mycobacterial disease resulting from MAC. Frequently, the number of organisms found in the bowel in these patients is quite high, although it has been reported that 68% of MAC culture-positive stool specimens are acid-fast-smear negative. Stool specimens should be collected in clean containers without any preservative and sent directly to the laboratory for processing. If processing within a few hours is not possible, the specimen should be frozen at -20°C until it is processed. Culture of feces for mycobacteria from patients other than those with or at risk for AIDS is usually not warranted.

Blood

Mycobacteremia, once considered rare, is now often seen in patients with AIDS but is seen less frequently in other immunocompromised hosts. Most infections are caused by MAC. Recovery of the organism from blood is associated with clinical disease. The Isolator lysis-centrifugation system (Wampole Laboratories, Cranbury, NJ) has been reliable and recommended for blood samples for many years. Alternative recovery systems include direct inoculation of blood into a MYCO/F bottle (BD Diagnostic Systems) or the BacT/Alert MB blood medium (bioMérieux, Durham, NC). The collection systems are considered equivalent, although the Isolator system allows quantitative analysis, which may be used to monitor therapy and evaluate prognosis.

Tissue and other body fluids

At times, tissue and other body fluids might be needed for microscopic examination and culture. Whenever possible, CSF specimens should be from large-volume lumbar punctures to increase diagnostic yield. Diagnosis of tuberculous meningitis is extremely difficult. Peritoneal (ascitic fluid) smears are also rarely positive for AFB. Culture of large volumes and inoculation of the specimen into mycobacterial liquid media can help maximize yield in dilute specimens.

When noninvasive techniques have failed to provide a diagnosis, surgical procedures may be considered. Specimens obtained from the lung, pericardium, lymph nodes, bones, joints, bowel, or liver may be appropriate. The tissue or fluid should be collected aseptically and placed in a sterile container. If the tissue is not processed immediately, a small amount (10 to 15 mL) of sterile saline should be added to prevent dehydration. It may be necessary to collect fluid containing fibrinogen (e.g., pleural, pericardial, peritoneal) in a container with an anticoagulant. The amount of liquid recommended for culture differs—2 mL for CSF, 3 to 5 mL

for exudates and pericardial and synovial fluids, and 10 to 15 mL for abdominal and chest fluids. Immediate processing of these samples is important. When tissue is collected, histologic evaluation may reveal caseating or noncaseating granuloma formation with the presence of multinucleated giant cells. These histologic changes are consistent with but not specific for mycobacterial disease.

Digestion and decontamination of specimens

To ensure optimal recovery of mycobacteria from clinical specimens, many specimens must be processed before inoculation onto culture media. Each step must be conducted with precision. Specimens from sterile body sites can simply be concentrated by centrifugation (if a large volume) and inoculated. However, specimens that may contain commensal bacteria should be decontaminated and then concentrated.

Many clinical specimens, such as sputum specimens, contain mucin or organic debris that surrounds the bacteria in the sample. An abundance of nonmycobacterial organisms, and possible mycobacteria, make up the microbiota of these specimens. When placed onto culture media, the abundant nonmycobacterial organisms can quickly overgrow the more slowly growing mycobacteria. The purposes of the digestion-decontamination process are to (1) liquefy the sample by digesting the proteinaceous material and (2) allow the chemical decontaminating agent to contact and kill the nonmycobacterial organisms. The high lipid content in the cell wall of mycobacteria makes them somewhat less susceptible to the killing action of various chemicals. After liquefaction of the specimen, the surviving mycobacteria can be concentrated with centrifugation. Additionally, liquefying the mucin enables the mycobacteria to contact and use the nutrients of the medium onto which they are subsequently inoculated.

Specimens that contain mucus and require digestion and decontamination include sputum, gastric washing, BAL, bronchial washing, and transtracheal aspirate specimens. Voided urine, autopsy tissue, abdominal fluid, and any contaminated fluid require decontamination. Specimens from normally sterile sites, such as blood, CSF, synovial fluid, and biopsy tissue from deep organs, do not require decontamination. Sterility should be strictly maintained in collection and transport. Stool decontamination is especially difficult and may require repeated attempts.

Decontamination and digestion agents

Each laboratory should maintain a proper balance between the rate of recovery of mycobacteria and suppression of contaminating bacterial growth. Failure to isolate mycobacteria from patients with signs and symptoms of classic mycobacterial disease may indicate that the decontamination is too harsh. However, if more than 5% of all specimens cultured are contaminated, the decontamination procedure may be inadequate. In general, a range that is considered acceptable in this delicate balance is between 2% and 5% of bacterially contaminated mycobacterial cultures. The bactericidal action of a decontaminating agent is influenced by the concentration of the chemical agent, exposure time, and temperature; therefore alterations in any of these factors can affect the bactericidal effect. The optimal decontamination procedure requires

an agent that is mild and yields growth of mycobacteria while controlling contaminants. The use of selective media may diminish the need for harsh decontamination procedures.

Sodium hydroxide

Sodium hydroxide (NaOH), at a concentration of 2%, 3%, or 4%, serves as a digestant and decontaminating agent. It is a commonly used decontaminant but must be used with caution because it is only slightly less harmful to the mycobacteria than to the contaminating organisms.

N-acetyl-L-cysteine

A combination of a liquefying agent, such as N-acetyl-L-cysteine (NALC) or dithiothreitol, plus NaOH is commonly used. The liquefying agent has no inhibitory effect on bacterial cells; however, liquefaction of the sample allows the decontaminating chemical to come into uniform contact with the contaminating bacteria more readily. When contamination can be controlled with a lower concentration of NaOH, the recovery of mycobacteria is indirectly improved using the milder procedure because fewer mycobacteria are lost in the process. The addition of chlorhexidine has improved the recovery of NTM from sputum samples from patients with cystic fibrosis because of the inhibition of *Pseudomonas aeruginosa*, which is found in the respiratory tract of 80% of these patients. See [Appendix D](#) on Evolve for the NALC-NaOH digestion-decontamination procedure.

Benzalkonium chloride

Another digestant-decontamination procedure uses benzalkonium chloride (Zephiran) combined with trisodium phosphate (TSP). TSP rapidly liquefies sputum but requires a long exposure time to decontaminate the specimen. Benzalkonium chloride shortens the exposure time and effectively destroys many contaminants, with little bactericidal effect on tubercle bacilli. The addition of phosphate buffer to digested specimens results in greater isolation of mycobacteria. Benzalkonium chloride is bacteriostatic for tubercle bacilli, necessitating neutralization before plating or the use of egg-based media to exploit its inherent neutralizing capacity.

Oxalic acid

Oxalic acid, 5%, can also be used to decontaminate specimens contaminated with *P. aeruginosa*. This method is reported to be better than other alkali decontamination procedures when *P. aeruginosa* and certain other contaminants are present. Oxalic acid-treated specimens can be used with the broth-based culture systems.

Concentration procedures

The specific gravity of the tubercle bacilli ranges from 0.79 to 1.07. Because of the low specific gravity of the AFB, a low centrifugal force has a buoyant rather than a sedimenting effect. Excess mucus will compound this phenomenon. Treatment with mucolytic agents, such as NALC, splits mucoprotein, allowing greater sedimentation of AFB. Concentration centrifugation speeds must be at least 3000 times the gravitational constant (*g*) to maximize recovery.

Lower *g* necessitates longer centrifugation time. The consequences of longer centrifugation time are prolonged exposure to the toxic effects of the digestion-decontamination agents

used and the higher temperatures generated by unrefrigerated centrifuges. In summary, the digestion-decontamination agent used, its concentration, the length of exposure of the specimen to the agent, and the centrifugation speed and temperature all affect the recovery of *Mycobacterium* spp.

Staining for acid-fast bacilli

When Gram-stained, *Mycobacterium* spp. do not stain or stain faintly, giving a beaded appearance because of irregular uptake of the stain caused by the increased lipid content of the cell wall. Acid-fast smears are prepared directly from clinical specimens and from digested, decontaminated, and concentrated specimens.

The conventional acid-fast staining methods, Ziehl-Neelsen and Kinyoun, use carbolfuchsin as the primary stain, acid-alcohol as a decolorizing agent, and a methylene blue counterstain. The Ziehl-Neelsen staining procedure involves the application of heat with the carbolfuchsin stain, whereas the Kinyoun acid-fast stain is a cold stain. The Ziehl-Neelsen method tends to provide more consistent results. Because of the potential cross-contamination of AFB from one smear to another, careful attention should be paid to the staining technique. Staining jars should not be used. Smears should not contact one another when placed on staining racks. Slides are examined using a $\times 100$ oil immersion objective on a light microscope for 15 minutes, viewing a minimum of 300 fields before a slide is called *negative*.

The **auramine stain** or **auramine-rhodamine fluorochrome stain** is more sensitive than the carbolfuchsin stains. About 18% of all culture-positive specimens have smears that are positive on the auramine-rhodamine stain but negative on the Kinyoun or Ziehl-Neelsen stain. In addition, smears can be screened at a lower magnification ($\times 250$ to $\times 400$), thus allowing more fields to be examined in a shorter time. A fluorescence microscope equipped with an appropriate filter system is needed for the examination of a fluorochrome-stained smear. The smear is examined under a mercury vapor lamp with a strong, blue-filtered light. Positive smears reveal bright, yellow-orange bacilli against a dark background. Smears being examined for AFB should be carefully examined with a minimum of 300 fields, and three horizontal sweeps of a smear that is 2 cm long and 1 cm wide should be performed. Less than 10% of the rapidly growing mycobacteria may be acid fast; they may not stain at all with fluorochrome stains. If rapidly growing mycobacteria are suspected, smears should be stained with carbolfuchsin and a weaker decolorizing process used.

Individuals with extensive disease shed large numbers of organisms. However, many individuals have subtle infections from which fewer organisms will be shed. Thus the overall sensitivity of the acid-fast smear ranges from 20% to 80%, depending on the extent of the infection. Even with concentration techniques, the number of organisms observed on a smear will be considerably less than the number of organisms seen on a smear from an individual with bacterial pneumonia. The CDC has made recommendations regarding the interpretation and reporting of acid-fast smears ([Table 26.2](#)). In the interpretation of a smear as positive for AFB, laboratory scientists must realize that organisms other than *Mycobacterium* such as *Nocardia* spp., *Legionella micdadei*, and *Rhodococcus* spp. might stain at least partially acid fast.

Table 26.2 Acid-fast smear interpretation

Number of acid-fast bacilli seen		Quantitative report
Carbolfuchsin stain, ×1000	Fluorochrome stain, ×450	
0	0	No acid-fast bacilli seen
1–2/300 fields	1–2/70 fields	Doubtful acid-fast bacilli seen; resubmit another specimen for examination
1–9/100 fields	1–2/70 fields	1 +
1–9/10 fields	2–18/50 fields	2 +
1–9/field	4–36/field	3 +
>9/field	>36/field	4 +

Modified from Kent, P. T. & Kubica, G. P. (1985). *Public health mycobacteriology: a guide for the level III laboratory*. Atlanta, GA: Centers for Disease Control and Prevention.

Culture media and isolation methods

Mycobacteria are strictly aerobic and grow more slowly than most bacteria pathogenic for humans. The generation time of mycobacteria is longer than 12 hours; *M. tuberculosis* has the longest replication time, at 20 to 22 hours. The rapidly growing species generally form colonies in 2 to 3 days, whereas most pathogenic mycobacteria require 2 to 6 weeks of incubation. The growth of *M. tuberculosis* is enhanced by an atmosphere of 5% to 10% CO₂. Mycobacteria have an optimal pH between 6.5 and 6.8 and grow better in higher humidity. Most human pathogenic mycobacteria grow best at temperatures of 35° to 37° C. However, when mycobacteria are suspected in skin and soft tissue specimens, a second set of culture media should be incubated at 25° to 33° C for the recovery of *M. haemophilum*, *M. marinum*, *M. ulcerans*, and *M. chelonae*. One of the mycobacteria pathogenic for humans, *M. genavense*, does not grow on media used routinely to isolate mycobacteria and requires extended incubation (6 to 8 weeks), whereas *M. leprae* fails to grow on artificial media.

The many different media available for the recovery of mycobacteria from a clinical specimen are variations of three general types (Table 26.3): egg-based media, serum albumin agar media, and liquid media. Within each general type, there are nonselective formulations and formulations that have been made selective by the addition of antimicrobial agents. Because some isolates do not grow on a particular agar and each type of culture medium offers certain advantages, a combination of culture media is generally recommended for primary isolation. The use of a solid-based medium, such as LJ medium, in combination with a liquid-based medium is recommended for routine culturing of specimens for the recovery of AFB. Current guidelines recommend that two or more types of media be used when attempting to recover mycobacteria.

Egg-based media

The basic ingredients in an inspissated egg medium, such as LJ (the most used egg-based medium), Petragnani, and American Thoracic Society (ATS) media, are fresh whole eggs, potato flour, and glycerol, with slight variations in defined salts, milk, and potato flour. Each contains malachite green to suppress the growth of gram-positive bacteria. Selective media that contain antimicrobial agents, such as the Gruft

modification of LJ medium and Mycobactosel (BD Diagnostic Systems), are sometimes used in combination with nonselective media to increase the isolation of mycobacteria from contaminated specimens. The nonselective egg-based media have a long shelf life up to 1 year, but distinguishing early growth from debris is sometimes difficult.

Agar-based media

Agar-based media are better chemically defined than egg-based media, and they do not readily support the growth of contaminants. Serum albumin agar media, such as Middlebrook 7H10 and 7H11 agars, are prepared from a basal medium of defined salts, vitamins, cofactors, glycerol, malachite green, and agar combined with enrichment consisting of oleic acid, bovine albumin, glucose, and beef catalase (Middlebrook OADC enrichment). **Middlebrook 7H11 medium** also contains 0.1% casein hydrolysate, which improves the recovery of isoniazid-resistant strains of *M. tuberculosis*. The addition of antimicrobial agents to 7H10 or 7H11 medium makes the media more selective by suppressing the growth of contaminating bacteria. Mitchison selective 7H11 agar contains polymyxin B, amphotericin B, carbenicillin, and trimethoprim lactate.

In contrast with opaque egg-based media, clear agar-based media can be examined using a dissecting microscope for early detection of growth and colony morphology. Drug susceptibility tests may be performed on agar-based media without altering drug concentrations, as occurs with egg-based media. When specimens are inoculated onto Middlebrook 7H10 and 7H11 media and incubated in an atmosphere of 10% CO₂ and 90% air, 99% of the positive cultures are detected in 3 to 4 weeks, earlier than for those detected on egg-based media. Certain precautions should be followed in the preparation, storage, and incubation of Middlebrook media. Both excess heat and exposure of the prepared media to light can result in the release of formaldehyde, which is toxic to mycobacterial growth.

A CHOC agar plate should be included in the primary isolation media for skin and other body surface specimens for the recovery of *M. haemophilum*, which requires ferric ammonium citrate or hemin for growth. Alternatively, a Middlebrook 7H10 agar plate supplemented with hemolyzed sheep red blood cells or another source of hemin may be used. The plate should be incubated at 30° C, the optimal temperature for recovery of this organism.

Table 26.3 Mycobacterial culture media

Medium	Composition	Inhibitory agents
American Trudeau Society	Fresh whole eggs, potato flour, glycerol	Malachite green (0.02%)
Petragnani	Fresh whole eggs, egg yolks, whole milk, potato, potato flour, glycerol	Malachite green (0.12%)
Middlebrook 7H9	Defined salts, vitamins, cofactors base, with pyruvate, albumin, catalase, glucose,	
Middlebrook 7H10	Defined salts, vitamins, cofactors, oleic acid, albumin, catalase, glycerol, glucose	Malachite green (0.00025%)
Middlebrook 7H11	Middlebrook 7H10 medium with 0.1% casein hydrolysate	Malachite green (0.0001%) Amphotericin B Nalidixic acid Trimethoprim Azlocillin Amphotericin B Nalidixic acid Trimethoprim Azlocillin
Gruft (modification of LJ)	Fresh whole eggs, defined salts, glycerol, potato flour, RNA	Malachite green Penicillin Nalidixic acid
Mycobactosel (BBL, BD Diagnostic Systems, Sparks, MD) LJ	Fresh whole eggs, defined salts, glycerol, potato flour	Malachite green Cycloheximide Lincomycin Nalidixic acid
Middlebrook 7H10 (selective)	Defined salts, vitamins, cofactors, oleic acid, albumin, catalase, glycerol, glucose	Malachite green Cycloheximide Lincomycin Nalidixic acid
Mitchison's selective 7H11	Middlebrook 7H10 medium with casein hydrolysate	Carbenicillin Amphotericin B Polymyxin B Trimethoprim lactate

LJ, Löwenstein-Jensen; SPS, sodium polyanethol sulfonate.

Liquid media

Mycobacterium spp. grow more rapidly in liquid medium, and it can be used for both primary isolation and subculturing. Liquid culturing systems have been demonstrated to be superior in the recovery of mycobacteria from clinical specimens compared with conventional solid media. Middlebrook 7H9 broth and Dubos Tween albumin broth are nonselective liquid media used for subculturing stock strains, picking single colonies, and preparing inoculum for in vitro testing. Numerous automated systems that use liquid media are available.

The mycobacterial growth indicator tube (MGIT) system (BD Diagnostic Systems) uses modified Middlebrook 7H9 broth with a fluorescence quenching-based oxygen sensor for detecting mycobacterial growth. Oxygen present in the sterile medium quenches the fluorescence. Bacterial growth consumes the oxygen, allowing fluorescence when the tube is exposed to UV light. In the manual system, a Wood lamp or

transilluminator can be used. Before use, oleic acid–albumin–dextrose is added to stimulate growth of mycobacteria. To inhibit growth of nonmycobacteria, the antimicrobial agents polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin (PANTA) are added. The MGIT 960 system (BD Diagnostic Systems) is a continuous monitoring system.

Other continuous monitoring systems include the MB/BacT Alert 3D system (bioMérieux), which uses a colorimetric carbon dioxide sensor in each bottle to detect bacterial growth, and the VersaTREK culture system (VersaTREK Diagnostic Systems, Cleveland, OH). The VersaTREK culture system is based on the detection of pressure changes in the headspace above the broth medium in a sealed culture bottle resulting from gas production or consumption during microbial growth. Each manufacturer provides a mixture of antimicrobial agents to be added to each culture vial at the time of inoculation. The continuous monitoring systems have similar

performance and operational features. Bottles are incubated in the instrument for the entire monitoring period, and options for electronic data management are available. The MGIT 960 and VersaTREK systems have also been approved for antimicrobial susceptibility testing. A disadvantage of the liquid systems is that no colony morphology or pigmentation is available to suggest that the growth is of a mycobacterial species and not that of a contaminant or commensal organism. Another limitation is that cultures with mycobacteria with a lower optimal temperature, such as *M. haemophilum*, *M. marinum*, *M. ulcerans*, and *M. chelonae*, may not be detected.

Isolator lysis-centrifugation system

Isolator is a blood collection system that contains saponin to liberate intracellular organisms. After saponin treatment and centrifugation, the sample is inoculated onto mycobacteria agar plates or tubes. The system allows higher yields and shorter recovery times for mycobacteria than conventional blood culture methods. It offers the advantage of yielding isolated colonies and the ability to quantify mycobacteremia, which may be useful in monitoring the effectiveness of therapy in disseminated MAC infection.

For maximal recovery of mycobacteria, laboratories should use a battery of media that includes a liquid medium and at least one solid medium—egg-based or agar-based—for primary isolation. An additional selective medium is often reserved for specimens in which heavy contamination is anticipated.

Case check 26.2

In the patient presented in the Case in Point, no organisms were seen on the direct smears of all three samples submitted. Nucleic acid amplification tests are a rapid, more sensitive method to directly detect mycobacteria. Cultures are more sensitive than direct smears, as well, for diagnosing TB, and broth cultures are preferred. The BACTEC broth cultures were ultimately positive in this patient.

Laboratory identification

Laboratory levels or extents of service

A change in the distribution of mycobacterial laboratory testing led to the development of levels of service by the ATS and extents of service by the College of American Pathologists (CAP) to maintain quality of service. According to CAP, laboratories must decide which level of mycobacterial services to offer: level 1, specimen collection only; level 2, perform microscopy and isolate and identify and sometimes perform susceptibility tests for *M. tuberculosis*; or level 3, perform microscopy, isolate and identify, and perform susceptibility tests for all *Mycobacterium* spp. (Box 26.3). A facility's selection of a level of service depends on the volume of specimens submitted, patient populations served, ability to perform the requested tests according to comfort; biological safety level and staff training in performance of each requested test; and the time, effort, and funds allocated for the service. The procedures available in a mycobacteriology laboratory differ with the level of service of that laboratory.

BOX 26.3 Levels of service as defined by the American Thoracic Society and College of American Pathologists

Extent of service as defined by the American Thoracic Society

- Specimen collection only; no mycobacteriologic procedures performed; all specimens sent to another laboratory
- Acid-fast stain, inoculation, or both; identification by a reference laboratory
- Isolation and definitive identification of *Mycobacterium tuberculosis*; preliminary grouping of nontuberculous *Mycobacterium* spp., with definitive identification at a reference laboratory
- Definitive identification of all mycobacterial isolates with assistance in the selection of therapy, with or without drug susceptibility testing

College of American Pathologists levels of service

- Level 1: Specimen collection only; no mycobacteriologic procedures performed; all specimens are sent to another laboratory
- Level 2: Perform microscopy. Isolate, identify, and sometimes perform susceptibility tests for *M. tuberculosis*
- Level 3: Perform microscopy. Isolate, identify, and perform susceptibility tests for all species of *Mycobacterium*

Preliminary identification of mycobacteria

Once an isolate has been recovered in the mycobacteriology laboratory, certain characteristics can be used to classify the isolate before performing biochemical or molecular tests. The first step is to confirm that the isolate recovered in broth or on solid media is an acid-fast organism by performing acid-fast staining. Then, phenotypic characteristics, such as colony morphology, growth rate, optimal growth temperature, and photoreactivity, can help speciate the mycobacteria. These characteristics do not allow definitive identification but are presumptive and help in the selection of other, more definitive tests. Historically, the Runyon classification used the rate of growth and pigment production to place the NTM into one of four categories. However, because of variability within individual species for these two criteria, the Runyon classification is no longer used today. Figs. 26.6 and 26.7 show schematic diagrams for the identification of slowly growing and rapidly growing *Mycobacterium* spp., respectively. See Table 26.1 for a summary of the identification characteristics of clinically important mycobacteria.

Colony morphology

Colonies of mycobacteria are generally distinguished as having a smooth and soft or rough and friable appearance. Colonies of *M. tuberculosis* that are rough often exhibit a prominent patterned texture referred to as *cording* (curved strands of bacilli); this texture is the result of tight cohesion of the bacilli. Colonies of MAC have a variable appearance, with glossy whitish colonies often occurring with smaller translucent colonies.

Growth rate

Growth rate and recovery time depend on the species of mycobacteria but are also influenced by the media, incubation temperature, and initial inoculum size. The range in recovery time is wide, from 3 to 60 days. Mycobacteria are generally categorized as rapid growers, having visible growth in fewer than 7 days, or slow growers, producing colonies in more than

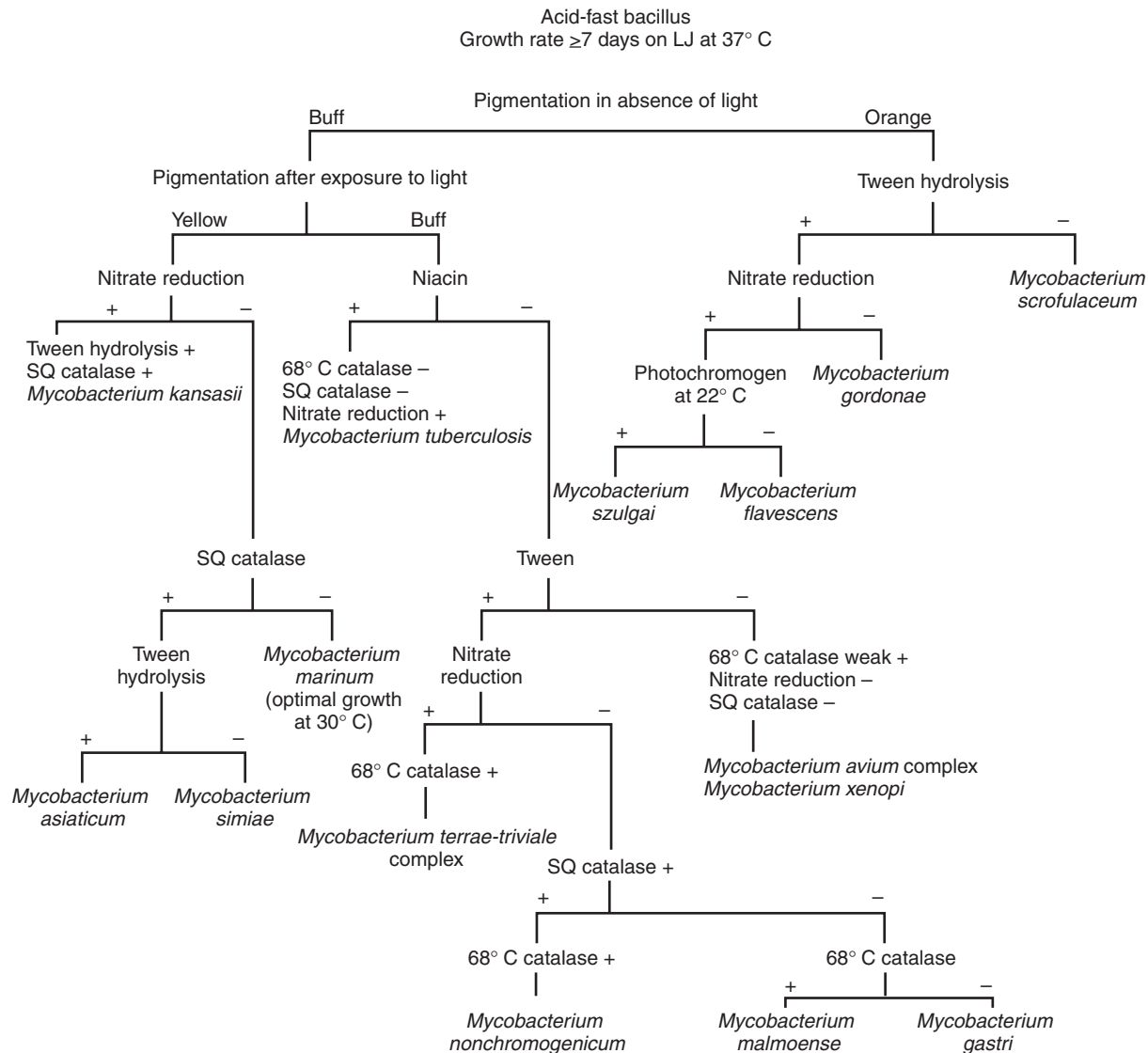


Fig. 26.6 Schematic diagram for the identification of slowly growing *Mycobacterium* spp. Exceptional reactions occur. Organisms should be subjected to a battery of morphologic and physiologic tests before final identification is made. LJ, Löwenstein-Jensen medium; SQ, semi-quantitative.

7 days. Determination of growth rate should be evaluated from the time of subculture, not from the time of detection from the clinical sample. The inoculum should be sufficiently small to produce isolated colonies. Microscopic examination of agar for microcolonies allows earlier detection of growth.

Temperature

The optimal temperature and range at which a mycobacterial species can grow may be extremely narrow, especially at the time of initial incubation. *M. haemophilum*, *M. marinum*, *M. ulcerans*, and *M. chelonae* grow best at 25° to 33° C and poorly, if at all, at 35° to 37° C. At the other extreme, *M. xenopi* grows best at 42° C.

Photoreactivity

Historically, *Mycobacterium* spp. have been categorized into three groups according to their photoreactive characteristics (Box 26.4). Species that produce carotene pigment on exposure to light are photochromogens (see Fig. 26.2). Color

ranges from pale yellow to orange. Species that produce pigment in the light and the dark are scotochromogens (see Fig. 26.4). Growth temperature may influence the photoreactive characteristics of a species. Pigment production is oxygen dependent, so it is important that tube caps are loose. Other species, such as *M. tuberculosis*, are nonchromogenic or non-photochromogenic (see Fig. 26.1). These colonies are a buff (tan) color and are nonphotoreactive; exposure to light does not induce pigment formation.

Biochemical identification

A panel of biochemical tests can identify most mycobacteria isolates, but because growth of most clinically significant *Mycobacterium* spp. is so slow, accomplishing this can take several weeks. Progress in molecular technology has diminished the frequency with which biochemical tests are routinely performed in the identification of mycobacteria. Because mycobacterial species may show only quantitative

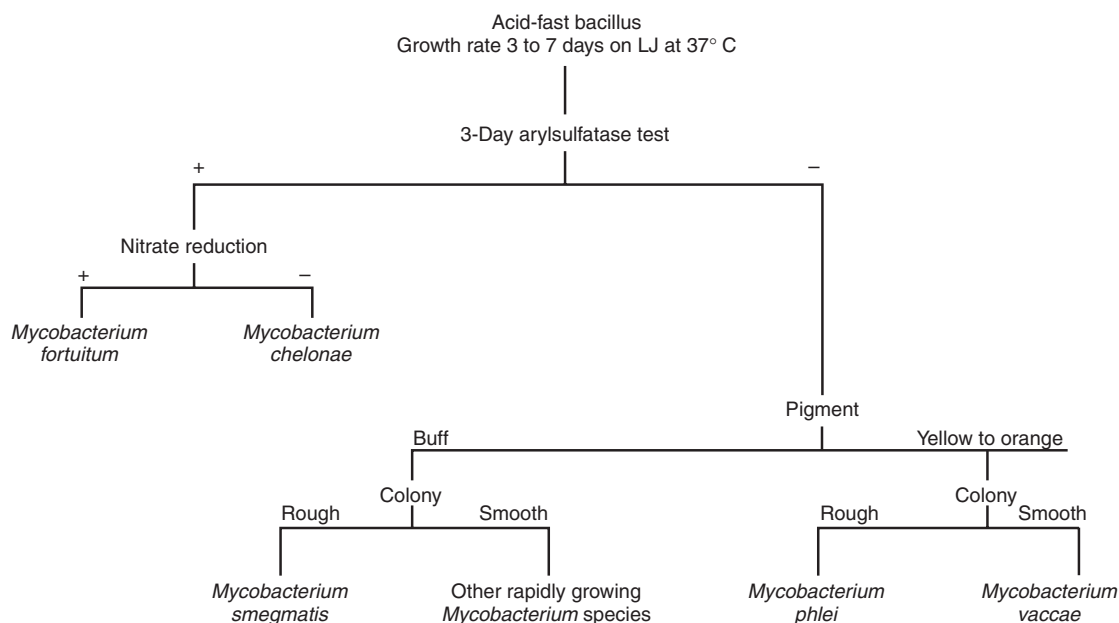


Fig. 26.7 Schematic diagram for the identification of rapidly growing *Mycobacterium* spp. Exceptional reactions occur. Organisms should be subjected to a battery of morphologic and physiologic tests before final identification is made. *LJ*, Löwenstein-Jensen medium.

BOX 26.4 Photoreactivity of clinically important mycobacteria

Nonchromogens

Slow growers

M. tuberculosis
M. avium complex^a
M. bovis
M. celatum
M. gastri
M. genavense
M. haemophilum
M. malmoeense
M. terrae complex
M. ulcerans

Rapid growers

M. chelonae
M. fortuitum group

Photochromogens

Slow growers

M. asiaticum
M. kansasii
M. marinum
M. simiae

Scotochromogens

Slow growers

M. gordonae
M. szulgai^b
M. scrofulaceum
M. xenopi^c

Rapid growers

M. phlei
M. smegmatis group

^aSome *M. avium* complex isolates are scotochromogens.

^bSome *M. szulgai* isolates are photochromogens.

^cYoung cultures may be nonchromogenic

differences in the enzymes used in biochemical identification, no single biochemical test should be relied on for the identification of a species. For expediency, all necessary biochemical tests should be set up at one time. The biochemical tests are based on enzymes that the organisms possess, the substances that their metabolism produces, and the inhibition of growth on exposure to selected biochemicals.

Niacin accumulation

Most mycobacteria possess the enzyme that converts free niacin to niacin ribonucleotide. However, 95% of *M. tuberculosis* isolates produce free niacin (nicotinic acid) because this species lacks the niacin-connecting enzyme. Accumulation of niacin, detected as nicotinic acid, was the most used biochemical test for the identification of *M. tuberculosis*. Nicotinic acid reacts with cyanogen bromide in the presence of an amine to form a yellow-pigmented compound. Reagent-impregnated strips eliminated the need to handle and dispose of cyanogen bromide, which is caustic and toxic. Because of molecular assays, this test is rarely used today.

The niacin test may be negative when performed on young cultures with few colonies. It is recommended that the test be done on egg agar cultures 3 to 4 weeks old (results are most consistent when the test is performed on egg-based media) and with at least 50 colonies. Tests that yield negative results may need to be repeated in several weeks. The test should not be performed on scotochromogenic or rapidly growing species because *M. simiae*, the BCG strain of *M. bovis*, *M. africanum*, *M. marinum*, *M. chelonae*, and *M. bovis*, may be positive, although this rarely occurs.

Nitrate reduction

The production of nitroreductase, which catalyzes the reduction of nitrate to nitrite, is relatively uncommon among *Mycobacterium* spp., but a positive result may be seen in *M. kansasii*, *M. szulgai*, *M. fortuitum*, and *M. tuberculosis*. Bacteria are incubated in 2 mL of sodium nitrate at 37° C for 2 hours. Hydrochloric acid (1:1 dilution in water), sulfanilamide, and

N-naphthylethylenediamine dihydrochloride are then added; if the bacteria reduce the nitrate to nitrite, a red coloration occurs (Fig. 26.8). When no color change develops, however, either no reaction occurred, or the reaction has gone beyond nitrite. The addition of zinc detects nitrate and results in a color change to pink in a true-negative reaction. Commercially available strips have simplified the assay. The nitrate reduction test differentiates *M. tuberculosis* from the scotochromogens and MAC.

Catalase

Catalase is an enzyme that splits hydrogen peroxide into water and oxygen. Mycobacteria are catalase positive. However, not all strains produce a positive reaction after the culture has been heated to 68° C for 20 minutes. Isolates that are catalase positive after heating have a heat-stable catalase (Fig. 26.9). Most MTBC organisms do not produce heat-stable catalase; exceptions are strains resistant to isoniazid. Other heat-stable, catalase-negative species include *M. gastri*, *M. haemophilum*, and *M. marinum*. Semi-quantitation of catalase production is based on the addition of Tween 80 (a detergent) and hydrogen peroxide to a 2-week-old culture grown in an agar deep. The reaction is read after 5 minutes, and the resulting column of bubbles is measured (Fig. 26.10). The column size is recorded as greater than or less than 45 mm.

Hydrolysis of Tween 80

Some mycobacteria possess a lipase that can split the detergent Tween 80 into oleic acid and polyoxyethylated sorbitol. The pH indicator neutral red is initially bound to Tween 80 and has an amber color. After hydrolysis of Tween 80, neutral red can no longer bind, and it is released, causing pink

coloration. The time required for the hydrolysis is variable. Results are recorded as positive after 24 hours, 5 days, or 10 days. This test is helpful in distinguishing between scotochromogenic and nonphotochromogenic mycobacteria.

Iron uptake

Some mycobacteria can convert ferric ammonium citrate to an iron oxide. After growth of the isolate appears on an egg-based medium slant, rusty brown colonies appear in a positive reaction on the addition of 20% aqueous solution of ferric ammonium citrate; coloration is the result of iron uptake (Fig. 26.11). The test is most useful in distinguishing *M. chelonae*, which is generally negative for iron uptake, from other rapid growers, which are positive.

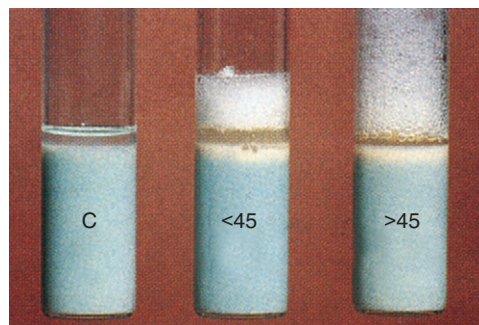


Fig. 26.10 Semi-quantitative catalase test.

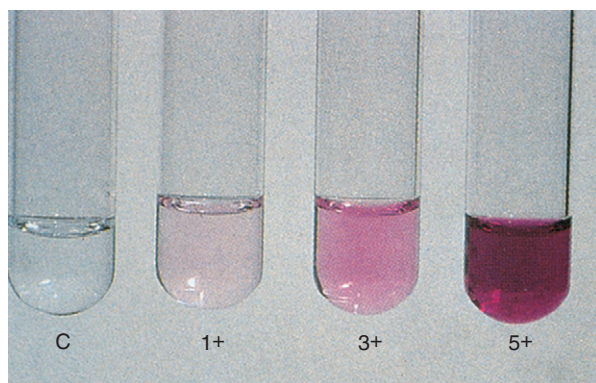


Fig. 26.8 Nitrate reduction test.

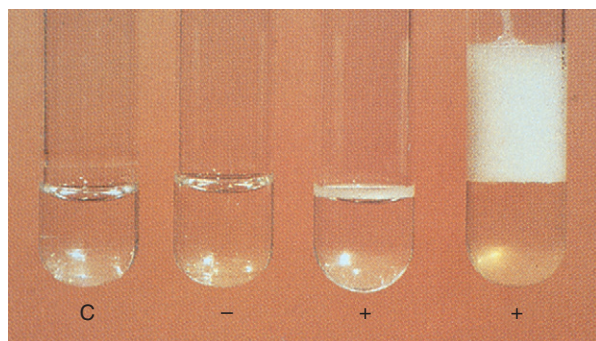


Fig. 26.9 Catalase test.



Fig. 26.11 Iron uptake.

Arylsulfatase

Most members of the genus *Mycobacterium* possess the enzyme arylsulfatase. This enzyme hydrolyzes the bond between the sulfate group and aromatic ring structure in compounds with the formula $R-OSO_3H$. Tripotassium phenolphthalein sulfate is such a molecule, from which phenolphthalein is liberated with exposure to arylsulfatase. The liberation of phenolphthalein causes a pH change in the presence of sodium bicarbonate, indicated by the change to a pink coloration. The *M. fortuitum* complex, *M. chelonae*, *M. xenopi*, and *M. triviale*, have rapid arylsulfatase activity that can be detected in 3 days. *M. marinum* and *M. szulgai* exhibit activity with 14 days of incubation.

Pyrazinamidase

Pyrazinamidase hydrolyzes PZA to pyrazinoic acid and ammonia in 4 days. Ferrous ammonium sulfate combines with pyrazinoic acid, producing a red pigment (Fig. 26.12). This reaction occurs in about 4 days and may be useful in distinguishing *M. marinum* (positive) from *M. kansasii* (negative) and *M. bovis* (negative) from *M. tuberculosis* (positive).

Tellurite reduction

Reduction of colorless potassium tellurite to black metallic tellurium in 3 to 4 days is a characteristic of MAC (Fig. 26.13) and thus is useful in distinguishing MAC from other nonchromogenic species. In addition, all rapid growers can reduce tellurite in 3 days.

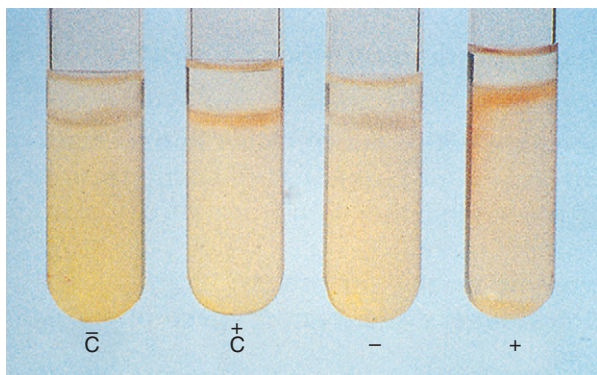


Fig. 26.12 Pyrazinamidase test.

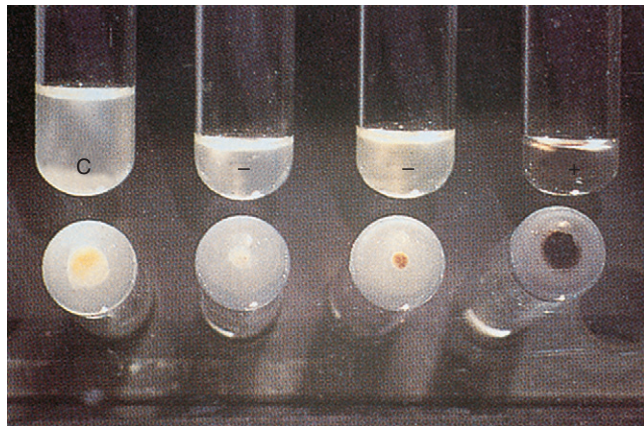


Fig. 26.13 Test for tellurite reduction.

Urease

Detection of urease activity can be used to distinguish *M. scrofulaceum*, which is urease positive, from *M. goodii*, which is urease negative (Fig. 26.14). A loopful of test organism is grown in 4 mL of urea broth at 37° C for 3 days. A pink-to-red color is indicative of a positive reaction.

Inhibitory tests

Thiophene-2-carboxylic acid hydrazide

T2H distinguishes *M. bovis* from *M. tuberculosis*. *M. bovis* is susceptible to lower concentrations of T2H than *M. tuberculosis*. Variability in inhibition exists, depending on the concentration of the inhibitory agent and the temperature of incubation.

Sodium chloride tolerance

High salt concentration (5% NaCl) in egg-based media (e.g., LJ medium) inhibits the growth of most mycobacteria. *M. flavescens*, *M. triviale*, and most rapidly growing *Mycobacterium* spp. are exceptions that do grow in the presence of 5% NaCl.

Growth on MacConkey agar

The *Mycobacterium fortuitum-chelonae* complex can grow on MacConkey agar without crystal violet, whereas most other mycobacteria cannot. This is not the same formulation of MacConkey agar typically used for the isolation of enteric bacilli.

Chromatography

The cell walls of *Mycobacterium* spp. contain long-chain fatty acids called *mycolic acids*, which can be detected by chromatography. The type and quantity of mycolic acids are species specific. Identification of *Mycobacterium* spp. uses HPLC (see Chapter 11). Enough mycolic acid can easily be extracted from small quantities of bacterial cultures. A basic saponification followed by acidification and chloroform extraction allows accurate identification of most mycobacterial species using chromatograms. Species identifications made with HPLC have been shown to agree well with biochemical and nucleic acid probe identifications. Chromatography is rapid and highly reproducible, but the initial equipment cost is high. This method has largely been replaced by molecular assays and MALDI-TOF mass spectrometry.

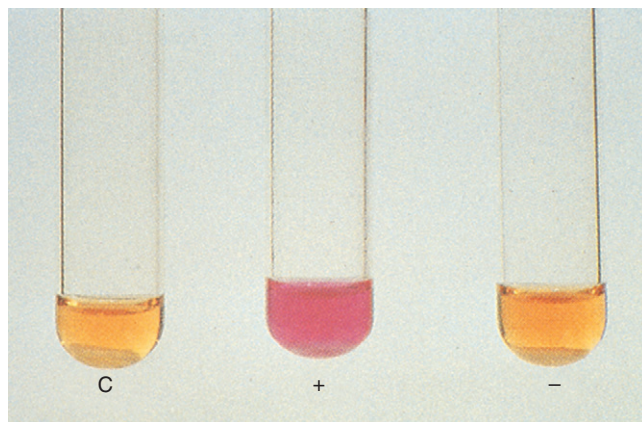


Fig. 26.14 Urease test.

Hybridization and nucleic acid amplification tests for *Mycobacterium tuberculosis*

The use of nucleic acid hybridization techniques allows the rapid identification of several common mycobacterial species. The first commercially available nucleic acid probe was the AccuProbe (Gen-Probe, San Diego, CA). It was approved for use on culture isolates to identify the MTBC, the MAC, *M. avium*, *M. intracellulare*, *M. kansasii*, and *M. goodii*. These tests use labeled (e.g., acridine ester–labeled nucleic acid) probes specific to mycobacterial ribosomal RNA (rRNA). The rRNA is released from the cell after sonication. The DNA probe is incubated with the test solution. If specific rRNA is present, a stable DNA-RNA complex, or hybrid, is formed. Unbound probe is chemically degraded. The complex is detected by adding an alkaline hydrogen peroxide solution. The hybrid-bound acridine ester is available to cause a chemiluminescent reaction, resulting in the emission of light. The amount of light emitted is related to the amount of hybridized probe.

The sensitivity of the assay ranges from 95% to 100%, depending on the species and species complexes. The amount of organism required for testing in the case of *M. tuberculosis* is a single colony of at least 1 mm in diameter, or in the case of MAC, a barely visible film of growth on the surface of the medium. Most positive results are well above the cutoff value of 10% hybridization. When a probe is used on a contaminated specimen, the resulting hybridization percentage may incorrectly fall below accepted cutoff hybridization levels, leading to a false-negative result. DNA hybridization identification can be applied to growth on conventional agar and to growth in liquid media. The combination of broth-based growth for detection and DNA hybridization identification using the probe technology allows rapid recovery and identification.

In addition to hybridization assays, many laboratories use nucleic acid amplification tests to identify mycobacteria. The INNO-LiPA Mycobacteria v2 test (Fujirebo, Malvern, PA) is a line probe assay that targets the 16S-23S rRNA spacer region of mycobacteria and has been used to directly detect and identify the MTBC, the MAC, *M. kansasii*, *M. xenopi*, *M. goodii*, and *M. chelonae*. The GenoType Mycobacteria Direct test (Hain Lifescience, Nehrin, Germany) uses a similar format and has additional probes for *M. celatum*, *M. mageritense*, *M. peregrinum*, and *M. fortuitum*. Although these assays have not yet received FDA approval, many U.S. laboratories have validated them.

Automated DNA sequencing is the most accurate method for the identification of mycobacterial isolates. A commonly used target is the gene coding for 16S rRNA. This gene is present in all bacteria and contains conserved and variable regions. Identification is accomplished by PCR amplification of DNA, followed by sequencing of the amplicons (amplified products). The organism is identified by comparison of the nucleotide sequence with reference sequences in a quality database. Other genes targeted include the *hsp65* and *rpoB* genes. Although this method holds great promise, sequences in some reference databases are not accurate, and procedures are not yet standardized. The FDA has not approved sequence databases, but some laboratories have verified sequencing tests as laboratory-developed tests. As molecular methods became more widely available commercially, identification and detection of mycobacteria has become faster and more specific.

Direct nucleic acid amplification tests

Nucleic acid amplification assays designed to detect MTBC bacilli directly from patient sputum specimens can be performed in as little as 6 hours on processed specimens and offer the promise of same-day reporting of results for detection and identification of *M. tuberculosis*. This methodology is recommended by the CDC as a standard of practice. However, the test is only recommended on patients with signs and symptoms of TB. The Amplified Mycobacterium tuberculosis Direct test (Hologic, Marlborough, MA) consists of transcription-mediated amplification of a specific 16S rRNA target performed at a constant temperature for the detection of MTBC rRNA in smear-positive and smear-negative respiratory specimens. The Cepheid Xpert MTB/RIF (Cepheid, Sunnyvale, CA) consists of PCR amplification of the 584-base pair region of the 16S rRNA gene sequence that uses molecular beacon probes.

Mass spectrometry

MALDI-TOF mass spectrometry is becoming more commonly used in microbiology laboratories (see Chapter 11) and is used in the identification of the mycobacteria. Because of the increased risk of laboratory-acquired infections when working with the mycobacteria, the processing of these isolates for MALDI-TOF mass spectrometry is different from that for other bacteria. In addition, because of the unique cell wall of the mycobacteria, an extraction step is required before the sample is added to the target plate. MALDI-TOF mass spectrometry requires a large biomass, so subculturing is often required. Because of the slow-growing nature of the MTBC, this can take 1 to 3 weeks. Another drawback to this method is that the MTBC cannot be identified to the species level. Using customized databases, some U.S. laboratories have reported the ability to speciate some of the rapidly growing mycobacteria. The two commercially available systems, VITEK MS (bioMérieux) and the MALDI Biotyper system (Bruker Daltonics, Billerica, MA), have databases that include the MTBC and NTM. The MALDI-TOF mass spectrometry system provides accurate identification of mycobacteria from solid media. Tests from liquid media have yielded variable results.

Susceptibility testing of *Mycobacterium tuberculosis*

Along with the increased incidence of mycobacterial disease, the development of multidrug-resistant strains of mycobacteria has been observed. For patients to receive appropriate therapy, the CDC recommends that when isolated, *M. tuberculosis* be tested for susceptibility to isoniazid, rifampin, ethambutol, and streptomycin. PZA should also be considered. Similarly, testing should be repeated if the patient's cultures for *M. tuberculosis* remain positive after 3 months of therapy. Susceptibility testing of mycobacteria requires meticulous technique and experienced personnel. Therefore laboratories with few *M. tuberculosis* isolates should consider sending isolates to a reference laboratory for susceptibility testing. Currently, two main methods are used for determining antimicrobial susceptibility of MTBC: culture-based drug

susceptibility testing and molecular drug susceptibility testing. The culture-based drug susceptibility method includes the agar proportion test, two broth systems, and the Sensititre microtiter dilution method (Thermo Fisher Scientific, Waltham, MA).

The agar proportion method is commonly used in the United States and Europe for all antimycobacterial drugs except PZA. The recommended medium is Middlebrook 7H10 agar supplemented with oleic acid–albumin–dextrose–catalase medium. The agar is prepared with two different concentrations of the drugs and dispensed into quadrant Petri dishes. A bacterial suspension equal to a McFarland #1 standard is prepared. The suspension is then diluted 10^{-2} and 10^{-4} , and the dilutions are plated to separate media. The two dilutions provide a set of plates that should be countable (i.e., 100 to 300 colony-forming units [CFU] on the control plate). Plates are incubated at 37° C. A control plate is set up for each drug tested so the number of colonies on the test quadrants can be counted and compared with the number on the control quadrant. If the test growth is less than 1% of the control growth, the organism is susceptible, and if greater, it is resistant. By this method, results can be obtained in 2 to 3 weeks, depending on the growth rate of the organism. In clinical correlations with in vitro data, if greater than 1% of a patient's bacilli are resistant to a particular drug, treatment fails. The test is often referred to as the *proportion method* because it allows one to predict the probability that 1% of the cells are resistant or not.

The two FDA-approved liquid methods to determine the susceptibility of *M. tuberculosis* to antimycobacterial agents are the MGIT 960 and VersaTREK. The broth methods use the principles of the agar proportion assay and employ a continuous monitoring system providing results more quickly than the agar method. Growth is indicated by the amount of fluorescence or gas measured by the BACTEC MGIT 960 or VersaTREK culture system, respectively. For each drug tested, a standardized inoculum is added to drug-free and drug-containing vials. The growth rate in the absence or presence of drug is then compared. Because it is not necessary to wait to visually detect growth, continuous monitoring system–based susceptibility results are usually available in 3 to 13 days. This compares favorably to about 21 days for the gold standard agar proportion method.

Molecular assays for drug-resistant mycobacteria can provide results in a few hours. The Cepheid Xpert MTB/RIF, discussed earlier, can be performed on unprocessed or processed sputum samples. It not only can detect the presence of MTBC, but it also can detect five separate mutations within the *rpoB* gene that indicate rifampin resistance. The GenoType MTBDRplus VER 2.0 (Hain Lifescience) can be used on clinical samples and culture isolates. It too can identify MTBC and detect mutations indicating resistance to rifampin and isoniazid. When a mutation is found, the CDC recommends gene sequencing to confirm.

Susceptibility testing of most NTM is not performed routinely. An exception is the rapidly growing mycobacteria. Broth microdilution, agar disk diffusion, and the Etest (bioMérieux) have been used for performing susceptibility testing on the rapid growers. The Clinical and Laboratory Standards Institute published guidelines in 2003 recommending broth dilution as the reference method for NTM.

POINTS TO REMEMBER

- Mycobacteria are important causes of human diseases, such as TB and Hansen disease.
- Mycobacteria have a unique cell wall, and special stains are required to visualize the microorganisms.
- Many *Mycobacterium* spp. are environmental microorganisms infrequently isolated from clinical specimens.
- Isolation of mycobacteria requires specific safety precautions, including laboratories with negative air pressure, biological safety cabinets, the use of respirators and other PPE, and electric incinerators instead of flame incinerators.
- Most pathogenic mycobacteria are slow growers, taking up to several weeks for isolation on artificial media.
- Some *Mycobacterium* spp. produce a pigment, which can be a helpful feature in their identification.
- Other tests for identification of mycobacteria include rate of growth, nitrate reduction, niacin production, presence of heat-stable catalase, and sensitivity to T2H. However, phenotypic identification is being replaced by molecular methods such as nucleic acid amplification.
- Antimicrobial susceptibility testing of the mycobacteria, while important, is technically demanding and should be performed only by experienced personnel.

LEARNING ASSESSMENT QUESTIONS

1. Which fluorescent stain(s) can be used to detect the mycobacteria?
 - a. Auramine-rhodamine
 - b. Kinyoun
 - c. Ziehl-Neelsen
 - d. Both b and c
2. A nonpigmented mycobacterium is isolated that reduces nitrate to nitrite and is niacin positive. Which organism would you suspect?
 - a. *Mycobacterium kansasii*
 - b. *Mycobacterium xenopi*
 - c. *Mycobacterium tuberculosis*
 - d. *Mycobacterium avium* complex (MAC)
3. Which statement is correct regarding the causative agent of Hansen disease?
 - a. It is highly contagious.
 - b. It readily grows on most mycobacterial media.
 - c. It grows best at core body temperature (37° C).
 - d. It does not grow in vitro.
4. The skin test for tuberculosis is characterized by which of the following?
 - a. Detects antibodies to mycobacterial antigens
 - b. Detects a cell-mediated immune response to mycobacterial antigens
 - c. Uses the bacillus Calmette-Guérin (BCG) strain as the antigen source
 - d. Both a and b
5. When hired to work in the mycobacteriology laboratory, a laboratory scientist had a nonreactive PPD skin test. Six months later, his PPD skin test was reactive. What does this likely indicate?
 - a. It was a false-positive result.
 - b. Tuberculosis was acquired before his hire.

- c. He acquired *Mycobacterium tuberculosis* infection between the first and second skin test.
- d. Not enough information is provided to determine.
6. A primary care provider suspects a patient has a mycobacterial soft tissue infection. At which temperature should the inoculated media be incubated?
 - a. 4° C
 - b. 28° C
 - c. 35° C
 - d. 42° C
7. When trying to isolate *Mycobacterium* spp., which of the following specimens should be decontaminated before plating?
 - a. Sputum
 - b. Lung biopsy
 - c. Lymph node biopsy
 - d. Pleural fluid
8. A physician provides material from a patient with a skin infection that has spread to the bone. After 3 days of incubations at 35° C on Middlebrook 7H10 medium, small, off-white colonies are seen. Which of the following organisms would you suspect?
 - a. *Mycobacterium ulcerans*
 - b. *Mycobacterium chelonae*
 - c. *Mycobacterium fortuitum* group
 - d. *Mycobacterium avium* complex
9. Microscopic examination of material from a cervical lymph node taken from a child with lymphadenitis reveals acid-fast bacilli. Which of the following organisms would you suspect?
 - a. *Mycobacterium bovis*
 - b. *Mycobacterium kansasii*
 - c. *Mycobacterium genavense*
 - d. *Mycobacterium avium* complex
10. What are the current recommendations for the identification of *Mycobacterium tuberculosis* in the clinical laboratory?
11. Why should mycobacterial infections be treated for 6 months or longer, and why is there a need to use multiple drugs when treating *M. tuberculosis* infections?
12. How do the various levels of mycobacterial laboratory testing differ, and why should smaller-volume laboratories consider not performing full identification and susceptibility testing on mycobacterial isolates?
13. What are the methods used to process clinical specimens for mycobacterial culture and the reasons specimens must be decontaminated and digested before culture?
14. With respect to laboratory technique in the isolation and identification of mycobacteria, what are some causes of false-negative and false-positive results?
15. What are the important safety considerations for laboratories attempting mycobacterial isolation and identification?

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Medically significant fungi

Connie F. Cañete-Gibas and Nathan P. Wiederhold

CHAPTER OUTLINE

General characteristics, 598

- Yeasts versus molds, 598
- Hyaline versus phaeoid, 599
- Dimorphism and polymorphism, 600
- Reproduction, 600

Taxonomy, 600

- Ascomycota, 600
- Basidiomycota, 601
- Mucorales, 601
- Fungi imperfecti, 601

Mycoses, 601

- Superficial mycoses, 602
- Cutaneous mycoses, 602
- Subcutaneous mycoses, 602
- Systemic mycoses, 602

Clinically significant species, 603

- Agents of superficial mycoses, 603
- Agents of cutaneous mycoses, 604
- Agents of subcutaneous mycoses, 607
- Agents of systemic mycoses, 610
- Agents of opportunistic mycoses, 615
- Agents of yeast infections, 622
- Pneumocystis, 624

Laboratory diagnosis of fungi, 626

- Safety issues, 626
- Specimen collection, handling, and transport, 626
- Direct microscopic examination of specimens, 628
- Isolation methods, 630
- Fungi identification, 631

Immunodiagnosis of fungal disease, 635

Antifungal susceptibility, 635

- Antifungal agents, 635
- Antifungal susceptibility testing, 635

Bibliography, 637

OBJECTIVES

After reading and studying this chapter, you should be able to:

1. Describe the general characteristics and structures of fungi.
2. Compare the cellular characteristics and morphology of fungi (eucaryotes) and bacteria (procaryotes).
3. Compare asexual and sexual reproduction of fungi.
4. List the divisions of fungi.
5. Identify the major causes of fungal infections.
6. Describe the common opportunistic saprobes associated with infections in immunocompromised hosts.
7. Characterize the following different types of mycoses, including the tissues they affect:
 - a. Superficial
 - b. Cutaneous
 - c. Subcutaneous
 - d. Systemic
 - e. Opportunistic saprobes
8. Analyze the appropriate specimen collection procedures, staining methods, and culture techniques used in the mycology laboratory.
9. Describe the key characteristics associated with the identification of the clinically significant fungi.
10. Evaluate the methods used to identify fungi.
11. Differentiate chromoblastomycosis from eumycotic mycetoma.
12. Develop a laboratory protocol for the identification of the clinically significant yeast.

KEY TERMS

Anamorph
Arthroconidium
Ascospores
Ascus
Blastoconidium
Conidium
Dermatophytes

Dimorphic fungi
Eumycotic mycetoma
Germ tube
Hyphae
Macroconidium
Microconidium
Mold

Mycelia
Mycoses
Onychomycosis
Polymorphic fungi
Pseudo-germ tube
Pseudohyphae
Rhinocerebral mucormycosis
Rhizoids

Saprobe
Sporangiophores
Sporangiospores
Synanamorphs
Teleomorph
Thrush
Yeast

Case in point

A 32-year-old female patient developed a fever 12 days after bone marrow transplantation. Broad-spectrum antimicrobial therapy was initiated, but the fever persisted. On day 17, the patient developed skin lesions across her body and lower extremities; a biopsy was performed. Microscopic evaluation of the tissue revealed hyphal elements. That same day, after 4 days of incubation, the patient's blood cultures were positive with a yeastlike colony. Although antifungal therapy was initiated, the patient died on day 22.

Issues to consider

After reading the patient's case history, consider:

- The fungi most likely to be implicated in this patient's infection
- Steps the laboratory will use to resolve the discrepancy between what is seen in the tissue slides and what grows from the blood culture
- The steps necessary to arrive at a final identification of this agent of disease

Fungi constitute an extremely diverse group of organisms and are generally classified as **molds** or **yeasts**. Some have been recognized as true pathogens, whereas others are only known as environmental **saprobies**, living on nonliving material. Fungi can cause mild infections, trigger allergic reactions, including asthma, and produce serious life-threatening disease. With the widespread use of chemotherapy, radiation therapy, and diseases such as acquired immunodeficiency syndrome (AIDS) that affect the immune system, the line between pathogen and saprobe has been blurred. The isolation of all organisms, especially in the immunocompromised patient, must initially be considered a significant finding and evaluated in light of the patient's history and physical examination results.

General characteristics

The characteristics of fungi differ from those of plants or bacteria. Like plants, fungi are eukaryotic; they possess a true nucleus, with a nuclear membrane and mitochondria whereas bacteria are prokaryotic, lacking these structures. Unlike plants, fungi lack chlorophyll and must absorb nutrients from the environment. In addition, fungal cell walls are made of chitin, whereas those of plants contain cellulose. Most fungi are obligate aerobes that grow best at a neutral pH, although they tolerate a wide pH range. Moisture is necessary for growth, but spores and conidia survive in dry conditions for extended lengths of time.

Yeasts versus molds

Yeasts are single vegetative cells that typically form a smooth, creamy, bacterial-like colony without aerial hyphae. Because their macroscopic and microscopic morphologies are similar, identification of yeasts is based primarily on biochemical testing and molecular diagnostic methods. Yeasts reproduce by budding or fission. Budding involves maturation of the bud to an independent **blastoconidium** (daughter cell), as shown in Fig. 27.1. This process involves lysis of the yeast cell wall so that a blastoconidium can form. As this structure enlarges, the nucleus of the parent cell undergoes mitosis. Once the new nucleus is passed into the daughter cell, a septum forms and the daughter cell breaks free. During fission, two cells of equal size are formed. These cells continue to grow from the tips of the cell and divide only after a medial fission is formed.

Most molds have a fuzzy or woolly appearance because of the formation of **mycelia** (Fig. 27.2). Mycelia are made up of many long strands of tubelike structures called **hyphae**, which are either aerial or vegetative. Aerial mycelia extend above the surface of the colony and are responsible for the fuzzy appearance. In addition, aerial mycelia support the reproductive structures that produce conidia. Conidia, in many cases, are used to identify different fungal genera. The vegetative mycelia extend downward into the medium to absorb nutrients.

Microscopic appearance often aids in the identification of molds. In some species, antler, racquet, rhizoid, or spiral hyphae are formed (Fig. 27.3A). Antler hyphae have swollen, branching tips that resemble moose antlers. Racquet hyphae contain enlarged, club-shaped areas. Spiral hyphae are tightly coiled. Rhizoids (see Fig. 27.3B), rootlike structures, might be seen in some members of the order Mucorales, the causative agents of mucormycosis, and their presence and placement can assist with identification. Frequently, when fungal hyphae are being described, they are referred to as *septate* or *sparsely septate*. Septate hyphae show frequent cross-walls occurring perpendicular to the outer walls of the hyphae (Fig. 27.4A), whereas sparsely septate hyphae have few cross-walls at irregular intervals (see Fig. 27.4B). The term *aseptate*, which means absence of septations, has historically been used to describe the hyphae of members of the order Mucorales. Microscopic examination of hyphae associated with the

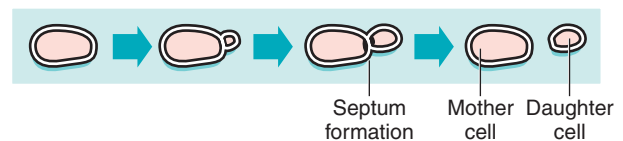


Fig. 27.1 Formation of blastoconidia in yeasts.

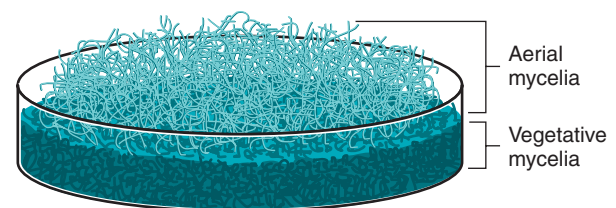


Fig. 27.2 Aerial mycelia give molds the “woolly” appearance. Vegetative mycelia are responsible for absorbing nutrients from the medium.

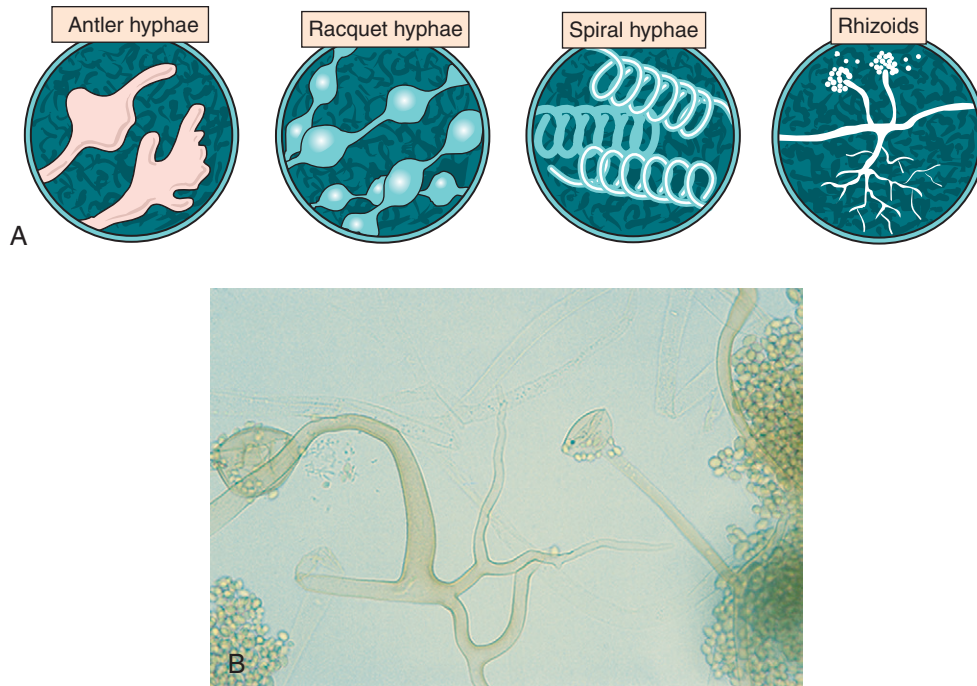


Fig. 27.3 **A**, Specialized structures formed in vegetative mycelia by certain fungal species. **B**, *Rhizopus* spp., showing rhizoids (unstained, $\times 200$).

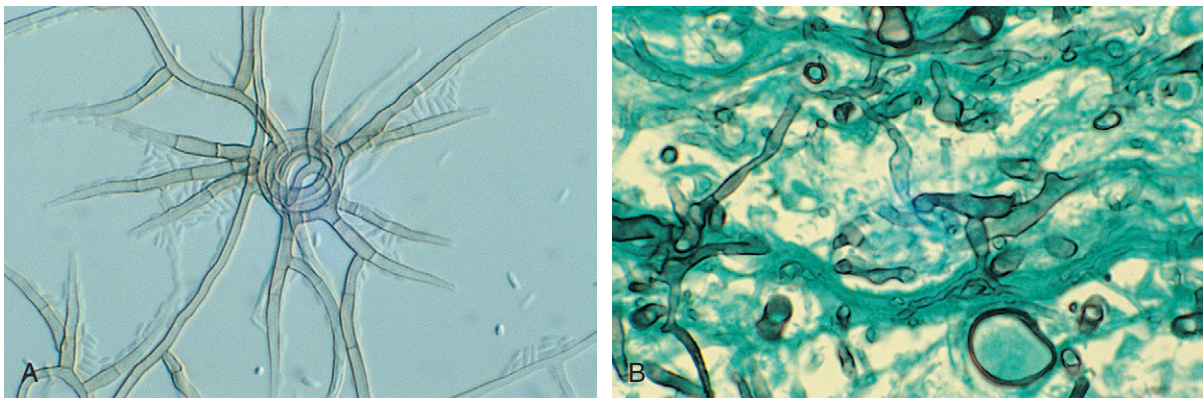


Fig. 27.4 **A**, *Phaeoacremonium* sp. displaying septate hyphae (unstained, $\times 200$). **B**, Mucorales hyphae in tissue appears sparsely septate (Gomori methenamine silver stain, $\times 400$).

Mucorales often reveals occasional septations; therefore these hyphae are more correctly termed *sparsely septate* as opposed to *aseptate*.

Hyaline versus phaeoid

Another useful characteristic in identification is pigmentation. Hyaline (moniliaceous) hyphae are nonpigmented or lightly pigmented, whereas phaeoid (dematiaceous) hyphae are darkly pigmented (Fig. 27.5) because of the presence of melanin in the cell wall. Depending on the amount of melanin present, the hyphae will appear pale to dark brown or almost black. Note that the dark hyphae seen in the tissue section in Fig. 27.4B is dark colored; however, it is because of the stain that enhances visualization of fungal elements in tissue and not because of melanin. All fungal elements appear black when Gomori methenamine silver (GMS) stain is used.



Fig. 27.5 *Bipolaris* sp. is an example of a phaeoid fungus. Note the dark pigmentation, which is caused by the presence of melanin in the cell wall (unstained, $\times 200$).

Another stain that is often used to determine hyphal pigmentation in tissue is the Fontana-Masson stain. This stain specifically stains melanin, causing phaeoid hyphae to appear brown, whereas hyaline hyphae stain pink to red.

Dimorphism and polymorphism

The term *dimorphism* refers to the ability of some fungi to exist in two forms, dependent on growth conditions. **Dimorphic fungi** include a mold phase and a yeast or spherule phase. The yeast or tissue state is seen *in vivo* or when the organism is grown at 37°C with increased concentration of carbon dioxide (CO₂). The mold phase is seen when the organism is grown at room temperature (22° to 25°C) in ambient air conditions. Thermally dimorphic fungal species associated with human disease include *Blastomyces* spp., *Coccidioides immitis/posadasii*, *Histoplasma* spp., *Paracoccidioides* spp., *Sporothrix* spp., and *Talaromyces* (formerly *Penicillium*) *marneffeii*. Several other fungi also possess this ability but have not been described as agents of human **mycoses** (infections caused by fungi). **Polymorphic fungi** have both yeast and mold forms in the same culture. This characteristic occurs despite growth conditions and is best observed in *Exophiala* spp., in which the yeast phase is typically observed initially, followed by the mold phase as the colony ages.

Reproduction

Fungi can reproduce asexually (imperfect) or sexually (perfect). Asexual reproduction results in the formation of conidia (singular, **conidium**) following mitosis. Asexual reproduction is carried out by specialized fruiting structures known as *conidiogenous cells*. These structures form conidia, which contain all the genetic material necessary to create a new fungal colony. Two common conidiogenous cells are the phialides and annellides. Phialides are vase-like structures that produce phialoconidia (Fig. 27.6), whereas annellides are ringed structures that produce annelloconidia. Both form their conidia blastically (budding) like many yeasts; the parent cell enlarges, and a septum forms to separate the conidial cell. Another type of conidia is the arthroconidia (singular, **arthroconidium**). These conidia are formed by fragmentation of fertile hyphae as opposed to being formed by conidiogenous cells (Fig. 27.7).

In the clinical laboratory, mold identifications may be based on microscopically observing structures formed as a result of asexual reproduction. Sexual reproduction requires the joining of two compatible nuclei, followed by meiosis (Fig. 27.8). A fungus that reproduces sexually is known as a **teleomorph**.

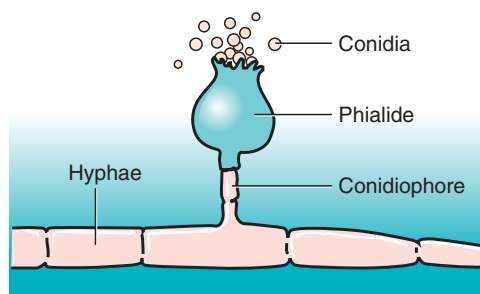


Fig. 27.6 An example of asexual reproduction is the production of phialoconidia. Conidia are formed from conidiogenous cells such as phialide (a vase-like structure). Phialoconidia are “blown out” of the phialide.

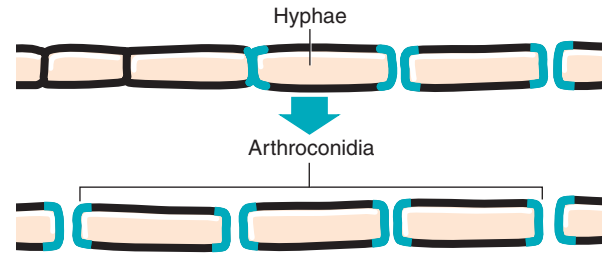


Fig. 27.7 In another form of asexual reproduction, arthroconidia are formed by fragmentation of fertile hyphae.

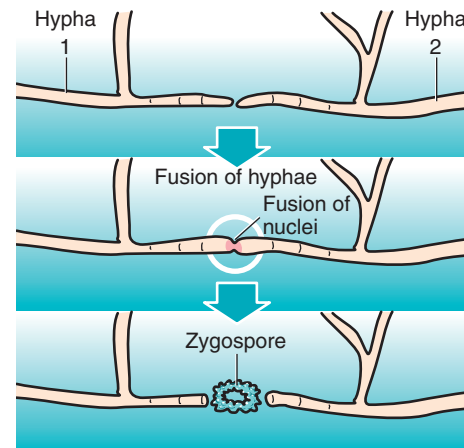


Fig. 27.8 Sexual reproduction occurs by the fusion of compatible nuclei and subsequent production of a zygospore.

Occasionally, these fungi will also reproduce asexually. When this occurs, the asexual form is termed the **anamorph**. If more than one anamorph is present for the same teleomorph, the anamorphic strains are termed **synanamorphs**.

Taxonomy

There are over 100,000 named fungal species and an estimated 1 million to 10 million undiscovered species. Most causative agents of clinical infections are found in four groups of fungi. They consist of the phyla Ascomycota and Basidiomycota, subphylum Mucoromycotina, and the form division Fungi Imperfecti (Deuteromycota).

Ascomycota

Approximately 50% of all named fungi are classified in the phylum Ascomycota. Fungi associated with the phylum Ascomycota are characterized by the production of sexual spores known as **ascospores**. Ascospores are formed within a sac-like structure known as an **ascus** (plural, asci). It is important to note, however, that they are usually identified on the basis of characteristic asexual structures. Representative organisms include *Microsporum* spp., *Trichophyton* spp., and *Scedosporium* spp. Phylogenetically, the genus *Candida* belongs to the phyla Ascomycota. However, many *Candida* species do not have a teleomorph; therefore they are categorized as Fungi Imperfecti.

Basidiomycota

Only a few members of the phylum Basidiomycota are clinically significant. The major pathogens are members of the *Cryptococcus neoformans* and *C. gattii* species complexes. Members of the genera *Malassezia*, *Trichosporon*, and *Rhodotorula* are also associated with human infections. Basidiomycetous molds are being recovered in increasing numbers in the laboratory, but their clinical significance is not clearly understood. Close communication between the physician and the laboratory would help determine whether the isolate is an environmental contaminant or an agent of disease.

When basidiomycetous molds are recovered in the laboratory, they typically remain sterile, complicating the identification process. One clue that a mold is a basidiomycete is the presence of clamp connections. Clamp connections occur at the septations in the vegetative hyphae and are easily visible under a light microscope. A portion of the hypha on one side of the septation grows out and connects to the hypha on the other side of the septation, thereby bypassing the septation. Clamp connections are not always present. Staining with diazonium blue B or growing on culture medium supplemented with benomyl can assist in the identification of a putative basidiomycete isolate.

Mucorales

The traditional Zygomycota have undergone taxonomic changes. Taxa traditionally placed in the phylum Zygomycota are now assigned among the phyla Mucoromycota, Basidiobolomycota, and Entomophthoromycota, subphyla Mucoromycotina, Basidiobolomycotina, and Entomophthoromycotina, respectively. The most clinically significant species belong to the subphylum Mucoromycotina consisting of the orders Mucorales, Umbelopsidales, and Endogonales and includes the genera *Actinomucor*, *Apophysomyces*, *Cokeromyces*, *Cunninghamella*, *Lichtheimia* (formerly *Absidia*), *Mucor*, *Rhizomucor*, *Rhizopus*, *Saksenaea*, and *Syncephalastrum*. Members of the order are rapidly growing organisms normally found in soil. They are often opportunistic pathogens in immunocompromised hosts. The clinical infection is referred to as *mucormycosis*.

Mucorales, the most clinically significant order, generally produce profuse, gray-to-white, aerial mycelia characterized by the presence of hyaline, sparsely septate hyphae. Asexual reproduction is characterized by the presence of **sporangiophores** and **sporangiospores**. The asexual spores (sporangiospores) are produced in a structure known as a *sporangium*, which develops from a supporting structure termed a *sporangiophore* (Fig. 27.9). Although some Mucorales are capable of sexual reproduction resulting in the production of zygospores, these structures are not routinely seen in clinical laboratories.

Fungi imperfecti

The form division Fungi Imperfecti contains the largest number of organisms that are causative agents of mycoses, including cutaneous, subcutaneous, and systemic diseases. Organisms are placed within this group when no mode of sexual reproduction has been identified. Therefore they are

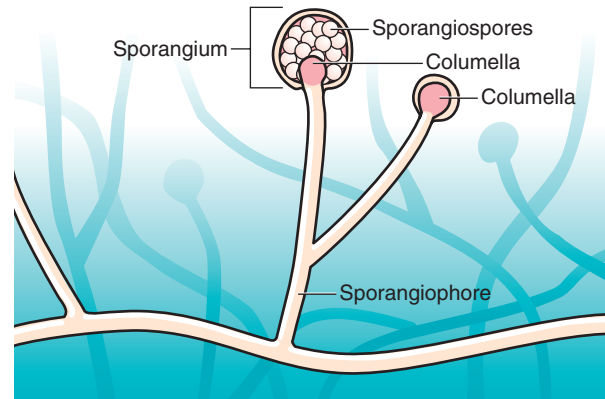


Fig. 27.9 Asexual reproduction by *Mucorales* is characterized by the production of spores (sporangiospores) from within a sporangium.

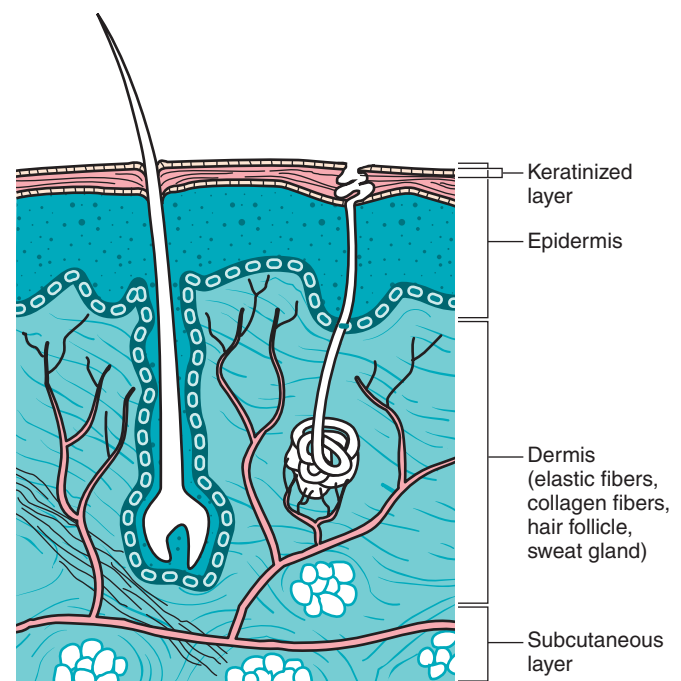


Fig. 27.10 The layers of skin and tissues in which fungal infections can occur.

identified on the basis of characteristic asexual reproductive structures.

Mycoses

Mycoses (singular, mycosis) are diseases caused by fungi. Fungal disease is frequently categorized on the basis of the site of the infection—superficial, cutaneous, subcutaneous, and systemic mycoses. Fig. 27.10 shows the different layers of tissues, which helps to classify infections of the skin, depending on where the infection occurs. Infections not involving the skin or deeper tissues just under the skin are termed *systemic*. Superficial and cutaneous mycoses are caused by fungi that can degrade keratin (dermatophytes) and fungi that cannot degrade keratin.

Superficial mycoses

Superficial mycoses are infections confined to the outermost layer of skin or hair. Because infections of skin, hair, and nails were at one time believed to be the result of burrowing worms that formed ring-shaped patterns in the skin, the term *tinea* (Latin, meaning “worm”) was applied to each disease, along with the Latin term for the body site. An example of nondermatophytic tinea is the disease tinea versicolor (pityriasis versicolor). This disease is characterized by discoloration or depigmentation and scaling of skin and is caused by the yeast *Malassezia furfur* complex. The disease becomes apparent in individuals with a dark complexion or in those who do not tan normally.

Another nondermatophytic superficial infection is tinea nigra. This disease is almost always caused by *Hortaea werneckii* and is characterized by brown or black macular patches, primarily on the palms. Biopsy and culture of the site are important to distinguish this infection from melanoma, a much more serious nonfungal disease. Another superficial infection, piedra, is confined to the hair shaft and is characterized by nodules composed of hyphae and a cementlike substance that attaches it to the hair shaft. Black piedra is caused by *Piedraia hortae*, and white piedra is caused by *Trichosporon ovoides* and *T. inkin*. Onychomycosis, caused by *Onychocola canadensis* and *O. kanei*, is also a nondermatophytic superficial mycosis characterized by white superficial and distal lateral subungual infections of the great toenail.

Cutaneous mycoses

Dermatormycoses are defined as fungal diseases of the keratinized tissues of humans and other animals. Although nondermatophyte species are capable of causing similar infections, this syndrome is usually a result of infection with a dermatophyte—hence, the term *dermatophytosis*. Note that this term should not be used until the causative agent has been identified as a dermatophyte. Genera in this group include *Trichophyton*, *Microsporum*, and *Epidermophyton*. Dermatophytic infections usually involve a restricted region of the host; traditionally, these diseases are named with respect to the portion of the body affected. The various forms of ringworm continue to be described in these terms, as shown in Table 27.1.

Table 27.1 Various forms of dermatophytoses and their respective affected sites	
Type of ringworm	Site affected
<i>Tinea capitis</i>	Head
<i>Tinea favosa</i>	Head (distinctive disease)
<i>Tinea barbae</i>	Beard
<i>Tinea corporis</i>	Body (glabrous skin)
<i>Tinea manuum</i>	Hand
<i>Tinea unguium</i>	Nails
<i>Tinea cruris</i>	Groin
<i>Tinea pedis</i>	Feet
<i>Tinea imbricate</i>	Body (distinctive lesion)

Each ringworm lesion is the result of local inoculation of skin with the causative agent. Lesions enlarge with time, usually with most inflammation occurring at the advancing edge of the lesion. However, some cases of ringworm are subclinical, exhibiting only a dry, scaly lesion without inflammation. Symptoms of cutaneous mycoses include itching, scaling, or ringlike patches on skin; brittle, broken hair; and thick, discolored nails.

Subcutaneous mycoses

Subcutaneous mycoses involve the deeper skin layers as well as muscle, connective tissue, and bone. Except in certain patient populations, dissemination through the blood to major organs does not occur. Characteristic clinical features include progressive, nonhealing ulcers and the presence of draining sinus tracts. In tropical areas, some agents, such as *Phialophora* spp. and *Cladosporium* spp., cause chromoblastomycosis, which is characterized by verrucous nodules that often become ulcerated and crusted. This disease is diagnosed by the presence of characteristic lesions accompanied by microscopic sclerotic bodies, often referred to as “copper pennies” because of their shape and staining properties in tissue sections.

Eumycotic mycetoma is caused by fungi and results in draining sinus tracts and tissue destruction. Grains (granules), which are tightly bound hyphae, can be collected from the fluids that drain from the sinus tracks and are useful in identifying the causative agent. The disease can be cutaneous or subcutaneous. Worldwide, about 40% of mycetomas are eumycotic, and the rest are actinomycotic, caused by *Actinomycetes* bacteria (see Chapter 16).

Sporotrichosis, caused by the *Sporothrix schenckii* species complex, commonly presents as a progressive lymphocutaneous infection, beginning with a single draining lesion and progressing along the limbs via the lymphatic system, forming multiple draining lesions. However, granules are not formed, thus this infection does not qualify as a mycetoma. Dissemination of these species causing systemic sporotrichosis is much more common, and *Sporothrix* has been implicated in pneumonia that is refractory to antifungal therapy.

Systemic mycoses

Systemic, or disseminated, mycoses are infections that affect internal organs or deep tissues of the body. Frequently, the initial site of infection is the lung, from which the organism disseminates hematogenously to other organs, including skin. Generalized symptoms include fever and fatigue. Chronic cough and chest pain might also accompany these infections. Historically, the term *systemic mycoses* was used to describe diseases caused by thermally dimorphic fungi, including *Histoplasma*, *Coccidioides*, and *Blastomyces* spp. However, other fungal agents are also capable of causing systemic disease, including but not limited to *Aspergillus*, *Fusarium*, *Scedosporium*, and *Curvularia* spp. as well as some yeasts, such as *Candida* and *Cryptococcus* spp. It is important to note that any fungus is capable of disseminating from the primary site of infection in the immunocompromised host.

Clinically significant species

Agents of superficial mycoses

Superficial mycoses are fungal diseases that affect only the cornified layers (stratum corneum) of the epidermis. Patients who have superficial fungal infections do not show any overt symptoms because the fungal agents do not activate any tissue response or inflammatory reaction. Patients usually seek medical attention to address cosmetic rather than medical concerns caused by these fungi.

Malassezia furfur

Clinical manifestations

The *Malassezia furfur* complex causes tinea versicolor, a disease characterized by patchy lesions or scaling of varied pigmentation. It is also thought to be a cause of dandruff. Lesions associated with tinea versicolor typically appear as pale patches in individuals with darkly pigmented skin, but they can also be described as fawn-colored liver spots in individuals with a fair complexion. Lesions become especially evident in warm months, when sun exposure is more likely. Tinea versicolor may involve any area of the body, but the most prevalent sites include the face, chest, trunk, and abdomen. Antifungal therapy is not typically indicated, but the appearance of lesions may be diminished by treatment with antidandruff shampoos. Interestingly, *M. furfur* complex has also been implicated in disseminated infections in patients receiving lipid replacement therapy (total parenteral nutrition), particularly in infants. Removal of indwelling feeding lines is usually sufficient to clear infections without using antifungal therapy.

M. furfur is a common endogenous skin colonizer. Although the reasons for overgrowth by *M. furfur* resulting in clinical manifestation are still unknown, it appears to be related to squamous cell turnover rates. This is evidenced by the higher incidence of tinea versicolor among persons receiving corticosteroid therapy, which decreases the rate of squamous epithelial cell turnover. Investigators have identified genetic influence, poor nourishment, and excessive sweating as other factors that contribute to the overgrowth of the organism on the skin. This organism is found worldwide, with the greatest prevalence in hot, humid, and tropical locations.

Laboratory diagnosis

M. furfur can be identified by microscopic examination of skin scrapings from characteristic lesions in a potassium hydroxide (KOH) preparation or by observing yellow fluorescence with a Wood lamp on examination of the infected body site. Microscopic examination of the direct smear in KOH preparations reveals budding yeasts, approximately 4 to 8 μm , along with septate, sometimes branched, hyphal elements. This microscopic appearance has gained *M. furfur* the nickname the spaghetti-and-meatballs fungus (Fig. 27.11).

M. furfur requires lipids for growth and will not grow on routine fungal media that have not been supplemented with a lipid source. Because of its special nutritional requirements, routine fungal cultures are negative for growth. Typical yeast-like colonies may be observed only after the culture medium has been overlaid with olive oil. Colonies are cream colored, moist, and smooth.

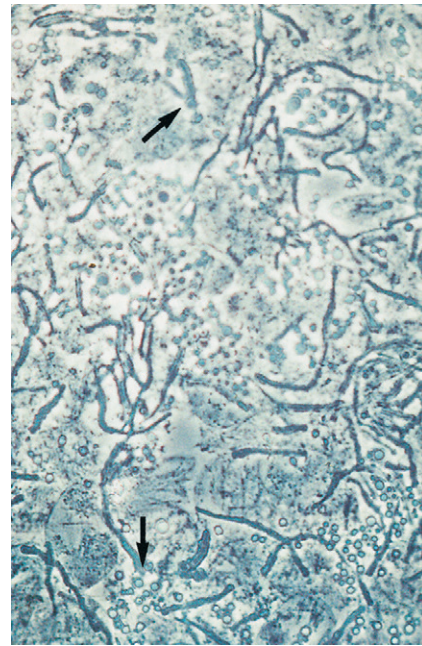


Fig. 27.11 The typical so-called spaghetti-and-meatballs appearance showing hypha (top arrow) and a yeast cell (bottom arrow) of *Malassezia furfur* in a potassium hydroxide preparation ($\times 400$).

Piedraia hortae

Clinical manifestations

Piedraia hortae is the causative agent of black piedra, an infection of scalp hairs. The infecting organism produces hard, dark brown to black gritty nodules that are firmly attached to the hair shaft. These nodules consist of asci (sac-like structures) containing eight ascospores. The disease is endemic in tropical areas of Africa, Asia, and Latin America.

Laboratory diagnosis

When infected hairs are removed and placed in 10% to 20% KOH, the nodules may be crushed open to reveal the asci. Thick-walled rhomboid cells containing ascospores are seen. *P. hortae* grows slowly on Sabouraud dextrose agar at room temperature. It forms brown, restricted colonies that remain sterile.

Trichosporon spp.

Clinical manifestations

Trichosporon beigelii was previously described as a human pathogen. However, examination of the genome of members of the genus has revealed that *T. beigelii* is no longer a valid species infecting humans. More than 30 distinct species exist, but *Trichosporon ovoides*, *T. asteroides*, *T. inkin*, and *Cutaneotrichosporon* (previously *Trichosporon*) *cutaneum* have been implicated in most cases of superficial mycoses. An important species in this genus is *T. asahii*, which is implicated in severe and frequently fatal disease in immunocompromised hosts. Although less frequently encountered, *T. mucoides* also causes systemic disease (meningitis) and is recovered frequently from cerebrospinal fluid (CSF). The colony resembles young colonies of *Cryptococcus neoformans* but

is easily differentiated on the basis of physiologic and morphologic characteristics. *Apiotrichum* (formerly *Trichosporon*) *mycotoxinovorans* is associated with pulmonary colonization and disease in patients with cystic fibrosis.

Trichosporon spp. are occasionally found as part of the normal skin biota and can also be isolated from animals and soil. White piedra occurs on the hair shaft and is characterized by a soft mycelial mat surrounding hair of the scalp, face, and pubic region. Members of this genus have also been recognized as opportunistic systemic pathogens. Although rare, systemic diseases caused by these fungi are frequently fatal and occur most often in immunocompromised hosts, commonly those who have hematologic disorders or malignancies or are undergoing chemotherapy. Infections in immunocompromised patients include infections of the blood, CSF, and organs. White piedra is endemic in tropical areas of South America, Africa, and parts of Asia.

Laboratory diagnosis

Trichosporon spp. grow rapidly on primary fungal media and produce arthroconidia, hyphae, and blastoconidia (Fig. 27.12). The colonies are straw- to cream-colored and yeast-like. Colonies are varied and can be smooth or wrinkled, dry or moist, and creamy or velvety in appearance. Identification to the species level is confirmed by absence of carbohydrate fermentation, use of potassium nitrate (KNO₃), assimilation of sugars, and urease positivity. Molecular and proteomic approaches, such as deoxyribonucleic acid (DNA) sequence analysis and matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry, respectively, in conjunction with biochemical reactions, lead to accurate identification of species within this genus.

Hortaea werneckii

Clinical manifestations

Tinea nigra, characterized by brown-to-black, nonscaly macules that occur most often on the palms and soles, is caused by *H. werneckii*. Obsolete synonyms for *H. werneckii* include *Phaeoannellomyces werneckii* and *Exophiala werneckii*. This disease involves no inflammatory or other tissue reaction to the infecting fungus. However, the clinical presentation is so similar to that of other conditions that misdiagnosis could occur,

resulting in unnecessary surgical procedures, especially when confused with malignant melanoma. The disease is endemic in the tropical areas of Central and South America, Africa, and Asia.

Laboratory diagnosis

Diagnosis of tinea nigra can be made by direct examination of skin scrapings placed in 10% to 20% KOH. Microscopic examination shows septate hyphal elements and budding cells (Fig. 27.13). Younger cultures are primarily composed of budding blastoconidia, whereas the older mycelial portion of the colony shows wide, profusely septate hyphae with blastoconidia in clusters. Anelloconidia are seen in older hyphal colonies. *H. werneckii* produces shiny, moist, yeastlike colonies that start with a brownish coloration that eventually turns olive to greenish black.

Agents of cutaneous mycoses

General characteristics

Three genera of fungi—*Trichophyton*, *Microsporum*, and *Epidermophyton*—are causative agents of dermatophytoses. Species within these genera, referred to as **dermatophytes**, are keratinophilic; that is, they are adapted to grow on hair, nails, and cutaneous layers of skin that contain the scleroprotein keratin. Infection of deep tissue by these fungi is rare, but occasionally extensive inflammation and nail bed involvement or disseminated disease may result.

Most agents of dermatophytoses live freely in the environment (geophilic), but a few have adapted almost exclusively to living on animals (zoophilic) or human tissues (anthropophilic), and they are rarely recovered from any other source. Distribution of these species is generally worldwide, although a few are found only in restricted geographic regions.

Dermatophytes typically form two sizes of reproductive cells, **macroconidium** or **microconidium**. Both of these are anamorphic or asexual conidia, and their distinctive size, shape, and surface features make them valuable structures for species identification. Some dermatophytes are known to also have teleomorphic stages in which ascospores are the reproductive cells. Teleomorphs in this group of organisms are not observed in routine laboratory studies of patient

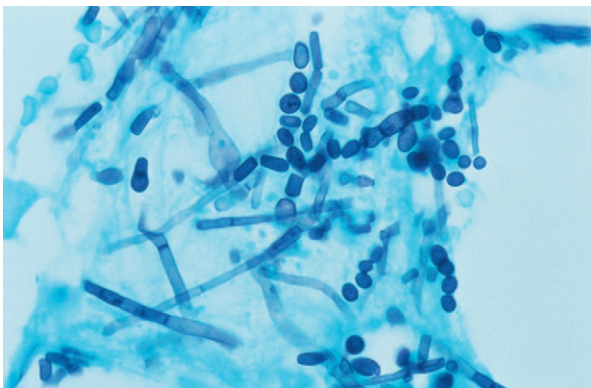


Fig. 27.12 Microscopic appearance of *Trichosporon* species on lactophenol cotton blue preparation showing the presence of both blastoconidia and arthroconidia ($\times 600$).

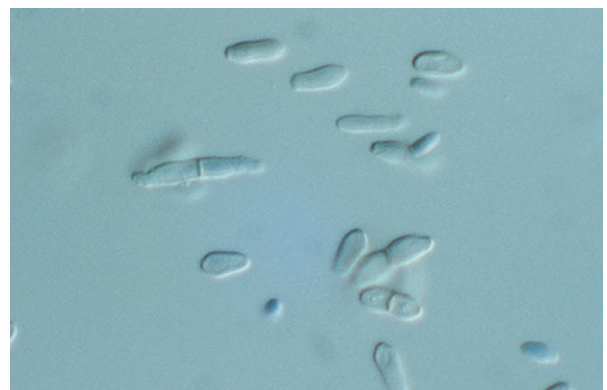


Fig. 27.13 Microscopic structures of *Hortaea werneckii*, showing characteristic budding annelloconidia ($\times 400$). *H. werneckii* causes tinea nigra.

isolates because dermatophytes are heterothallic, requiring the combination of two distinct mating types. Although a few reference laboratories perform mating tests with known *tester strains*, this procedure is not regularly performed in clinical laboratories.

Most geophilic fungi produce large numbers of conidia and therefore are among the most readily identified species. Zoophilic dermatophytes are not commonly found living freely in soil or on dead organic substrates. They often cause infections in animals and can be spread as agents of disease in humans. Fewer conidia are produced by zoophilic fungi than by geophilic species. Although the anthropophilic species are almost always encountered as agents of human disease, the infections are seldom inflammatory. Species identification may be difficult because most anthropophilic species produce few conidia.

Not only are sites diverse on the host involved in infections, but certain species also cause distinctive lesions. The notable example is tinea imbricata, caused by *Trichophyton concentricum*. Over time, involved portions of the trunk develop diagnostically distinctive concentric rings of scaling tissue. In some forms of ringworm, a persistent allergic reaction, dermatophytid, is manifested in the formation of sterile, itching lesions on body sites distant from the point of infection. Symptoms of dermatophyte infections range from slight to moderate and occasionally severe.

Infections involving hair

Different body sites manifest different symptoms of mycoses. Infections of the scalp, in which hair follicles are the initiation sites, can be among the most severe and disfiguring forms of mycoses. Tinea favosa, or favus, begins as an infection of the hair follicle by *Trichophyton schoenleinii* and progresses to a crusty lesion made up of dead epithelial cells and fungal mycelia. Crusty, cup-shaped flakes, called *scutula*, are formed. Hair loss and scar tissue formation commonly follow.

Two distinct forms of tinea capitis—gray patch ringworm and black dot ringworm—are caused by different species of dermatophytes. Gray patch ringworm is a common childhood disease that is easily spread among children. The fungus colonizes primarily the outer portion of hair shafts, the so-called *ectothrix* hair involvement. The lesions are seldom inflamed, but luster and color of the hair shaft may be lost. *Microsporum audouinii* and *Microsporum ferrugineum* are causative agents of this disease. Black dot ringworm consists of endothrix hair involvement. The hair follicle is the initial site of infection, and fungal growth continues within the hair shaft, causing it to weaken. The brittle, infected hair shafts break off at the scalp, leaving the black dot stubs. *Trichophyton tonsurans* and *Trichophyton violaceum* are the most common fungi implicated in this form of dermatophytosis.

Infections involving nails

Onychomycosis, which is infection of the nails, is most often caused by dermatophytes but also may be the result of infection by other fungi. These nail and nail bed infections may be among the most difficult dermatomycoses to treat. Long-term, costly therapy with terbinafine or itraconazole has been considered the best treatment, but results are often unsatisfactory. There is a direct association between dermatophytic

infections of the feet or hands and infections of the nails. It is unlikely that anyone suffering from tinea pedis will escape some extent of onychomycosis. The subungual form is the most common form of onychomycosis, described as lateral, distal, or proximal. Either end of the nail is first infected, with spread continuing to the nail plate. Nails become thick, discolored, and flaky. Some common agents that infect the nails are *Trichophyton rubrum*, *T. mentagrophytes*, and *T. tonsurans*, as well as *Epidermophyton floccosum*.

Tinea pedis

Among the shoe-wearing human population, tinea pedis (athlete's foot) is a common disease. Infection arises from infected skin scales coming into contact with exposed skin via a carpet, shower floors, or other shared walking/standing surfaces where shoes are not always worn. It is believed that individuals have a genetic predisposition to developing the disease because not everyone encountering infected skin scales becomes infected. Infections within a family are common. Various sites on the foot may be involved, but tinea pedis usually affects the soles and toe webs. In more severe cases, the sole may develop extensive scaling, with fissuring and erythema. The disease may progress around the sides of the foot from the sole, giving rise to use of the term *moccasin foot*, descriptive of the appearance of the infected area. Infections of the glabrous skin range from mild, with only minimal scaling and erythema, to severely inflamed lesions.

Systemic dermatophyte infections

Immunocompromised persons may suffer systemic dermatophyte infections. Disseminated disease appears in different forms. In some patients, it manifests itself as granulomas, whereas in others, pea-sized to walnut-sized nodules can develop. Biopsy of these nodules reveals fungal elements that are easily recovered in culture. This phenomenon has been seen primarily in kidney transplant recipients and is thought to have spread from athlete's foot or onychomycosis.

Epidermophyton floccosum

Epidermophyton floccosum produces only one size of conidia described as macroconidia. The smooth, thin-walled macroconidia are produced in clusters or singly. The distal end of the conidium is broad or spatulate and reminiscent of a beaver's tail. Occasionally, conidia may be single celled, but usually they are separated into two to five cells by perpendicular cross-walls.

Colonies of *E. floccosum* are yellow to yellow-tan, flat with feathered edges, and remain small in diameter. *Epidermophyton* isolates are notorious for developing pleomorphic tufts of sterile hyphae in older cultures. *Epidermophyton* is distributed worldwide.

Microsporum canis

Macroconidia from *M. canis* are spindle shaped, with echinulate, thick walls; they measure 12 to 25 μm $\times$ 35 to 110 μm and have 3 to 15 cells (Fig. 27.14). The tapering, sometimes elongated, spiny distal ends of the macroconidia are key features

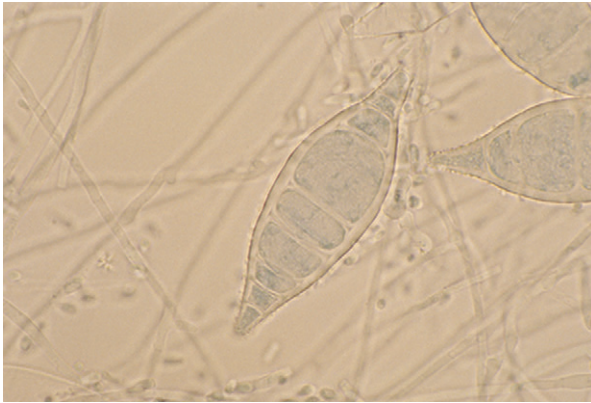


Fig. 27.14 *Microsporum canis* showing spindle-shaped, echinulate macroconidia with thick walls and tapered ends, which are key features in identification of this species (unstained, $\times 450$).

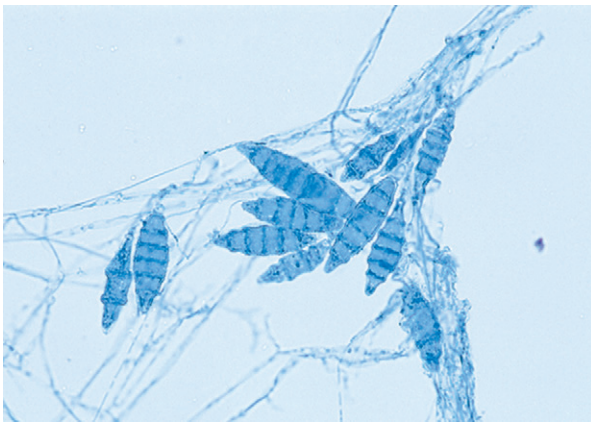


Fig. 27.15 *Nannizzia gypsea* (formerly *Microsporum gypseum*) showing fusiform, moderately thick-walled macroconidia containing several cells (lactophenol cotton blue preparation, $\times 450$).

that distinguish this species. Microconidia are abundantly formed by most isolates, and these may be the only conidia maintained in cultures that have been serially transferred. Colonies are fluffy and white, with the reverse side of the colony usually developing a lemon yellow pigment, especially on potato dextrose agar. This fungus has a worldwide distribution.

Nannizzia gypsea (formerly *Microsporum gypseum*)

The fusiform, moderately thick-walled conidia (Fig. 27.15) typical of *Nannizzia gypsea* measure 8 to $15\mu\text{m} \times 25$ to $60\mu\text{m}$ and can have as many as six cells. In some isolates, the distal end of the macroconidium might bear a thin, filamentous tail. Abundant macroconidia and microconidia produced by most isolates of this species result in a powdery, granular appearance on colony surfaces. Fresh isolates typically form tan to buff conidial masses, but this species tends to develop pleomorphic tufts of white sterile hyphae in aging cultures and after serial transfers. Abundant brown-to-red pigment can form beneath some strains, but others remain colorless. *N. gypsea* is a rapidly growing geophilic species found in soils worldwide.



Fig. 27.16 *Trichophyton mentagrophytes* showing globose, teardrop-shaped microconidia (Nomarski optics, $\times 450$).

Microsporum audouinii

A slow-growing anthropomorphic dermatophyte, *Microsporum audouinii* was responsible for most of the gray patch tinea capitis of children until a few decades ago, when *T. tonsurans* replaced it as the leading cause of scalp infection. In culture, conidia are only rarely produced. Some isolates form chlamydoconidium-like swellings terminally on hyphae. Colonies of *M. audouinii* appear cottony white and generally form little or no pigment on the reverse.

Trichophyton mentagrophytes

The microconidia (Fig. 27.16) of *Trichophyton mentagrophytes* are primarily globose but may appear tear shaped and measure 2.5 to $4\mu\text{m}$ in diameter. Microconidia are found primarily in clusters described as grapelike (“en grappe”). Macroconidia are thin walled, smooth, and cigar shaped, with four to five cells separated by parallel cross-walls. These conidia measure about $7\mu\text{m} \times 20$ to $50\mu\text{m}$ and are produced singly on undifferentiated hyphae.

Colony morphology varies with the extent of conidia production. Granular colonies are noted when abundant microconidia are formed. In the downy form, conidia are less abundant. Compared with other dermatophytes, *T. mentagrophytes* is a relatively rapidly growing fungus. This species is distributed worldwide.

Trichophyton rubrum

Although *Trichophyton rubrum* is known to produce three- to eight-celled cylindric macroconidia measuring somewhat smaller than those of *T. mentagrophytes*, these are seldom seen in clinical isolates. Typical microscopic appearance of *T. rubrum* reveals clavate- or peg-shaped microconidia (Fig. 27.17) formed along undifferentiated hyphae, and even these may be sparse. Colonies usually remain white on the surface but may be yellow to red. Most strains develop a red to deep burgundy wine-colored pigment on the reverse that diffuses into the agar. This species has a worldwide distribution.

Trichophyton tonsurans

Trichophyton tonsurans possesses microconidia that are extremely variable in shape, ranging from a round shape to a peg shape. When grown on Sabouraud dextrose agar,

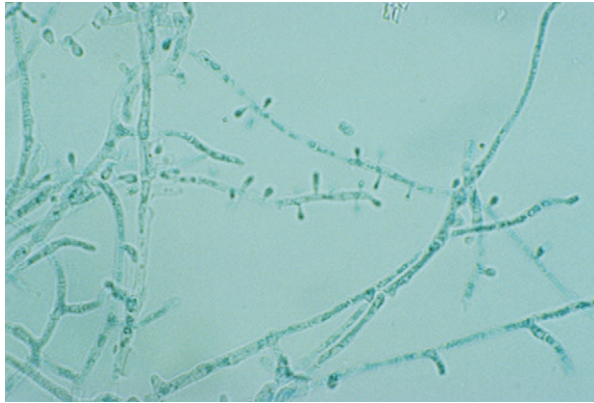


Fig. 27.17 *Trichophyton rubrum* showing clavate- or peg-shaped microconidia (lactophenol cotton blue preparation, ×450).

colonies usually form a rust-colored pigment on the colony's reverse. *T. tonsurans*, which infects skin, hair, and nails, has become the leading cause of tinea capitis in children in many parts of the world, including the United States.

Agents of subcutaneous mycoses

Subcutaneous mycoses are fungal diseases that affect subcutaneous tissue. These mycoses are usually the result of traumatic implantation of foreign objects into the deep layers of skin, permitting the fungus to gain entry into the host. The causative agents responsible are organisms commonly found in soil or on decaying vegetation; thus agricultural workers are most often affected. Organisms causing subcutaneous mycoses belong to a variety of genera in the form class Hyphomycetes. Although some are moniliaceous (hyaline or light colored), many are phaeoid, producing darkly pigmented colonies and containing melanin in their cell walls. The infections are commonly chronic and usually incite the development of lesions at the site of trauma. Subcutaneous fungal infections may be grouped together by the disease processes they cause or by the causative agents involved.

Chromoblastomycosis

Also known as *verruccous dermatitis* and *chromomycosis*, chromoblastomycosis occurs worldwide but is most common in tropical and subtropical regions of the Americas and Africa. In the United States, most cases occur in Texas and Louisiana. Several organisms are responsible for the disease, and certain organisms appear to reside in specific endemic areas throughout the world. Chromoblastomycosis is caused by several infectious agents, namely, *Fonsecaea pedrosoi*, *Phialophora verrucosa*, *Cladophialophora carrionii*, and *Rhinocladiella aquaspersa*.

Clinical manifestations

Chromoblastomycosis, one of the more common subcutaneous mycoses, is a chronic infection of skin and subcutaneous tissue and develops over a period of months or, more commonly, years. It is mostly asymptomatic in the absence of secondary complications, such as bacterial infections, carcinomatous degeneration, and elephantiasis. Lesions are usually confined to the extremities, often the feet and lower legs, and are a result of trauma to these areas. Lesions of

Table 27.2 Microscopic morphology of fungi causing chromoblastomycosis

Organism	Microscopic morphology
<i>Phialophora verrucosa</i>	Conidiogenous cells, phaeoid, flask-shaped phialides, with collarettes Conidia oval, one celled, occur in balls at tips of phialides
<i>Fonsecaea pedrosoi</i>	Primary one-celled conidia formed on sympodial conidiophores Primary conidia function as conidiogenous cells to form secondary one-celled conidia Some conidia are similar to those seen in <i>Cladophialophora</i> spp., some are similar to those in <i>Rhinocladiella</i> spp., and some are similar to those in <i>Phialophora</i> spp.
<i>Fonsecaea compactum</i>	Considered conspecific with <i>F. pedrosoi</i> . Similar to <i>F. pedrosoi</i> but with more compact conidial heads Conidia are subglobose rather than ovoid
<i>Cladophialophora carrionii</i>	Erect conidiophores bearing branched chains of one-celled, brown blastoconidia Conidium close to tip of conidiophore, termed <i>shield cell</i> Fragile chains
<i>Rhinocladiella aquaspersa</i>	Conidiophores erect, dark, bearing conidia only on upper portion near the tip Conidia elliptic, one celled, produced sympodially

chromoblastomycosis frequently appear as verrucous nodules that may become ulcerated and crusted. Longstanding lesions have a cauliflower-like surface. Brown, round sclerotic bodies, which are nonbudding structures occurring singly or in clusters, are seen in tissues. These sclerotic bodies reproduce by dividing in various planes, resulting in multicellular forms. Occasionally, short hyphal elements are also seen. The presence of sclerotic bodies is diagnostic for this disease. Males are predominately infected, possibly indicating a role of sex hormones in disease development. *Fonsecaea*, *Cladophialophora carrionii*, and *Phialophora verrucosa* cause most cases of chromoblastomycosis.

Laboratory diagnosis

The microscopic morphology of each of the agents is described in Table 27.2. *P. verrucosa* and *C. carrionii* are shown in Fig. 27.18. Isolates are identified on the basis of characteristic structures, such as arrangement of conidia and the manner in which conidia are borne. Organisms causing chromoblastomycosis are darkly pigmented. Growth is moderate to slow, and colonies are velvety to woolly and gray-brown to olivaceous black. Species are not differentiated by colony morphologies because they all produce similar characteristics. Molecular approaches, such as ribosomal gene sequencing, provide accurate identification to the species level.

Eumycotic mycetomas

Mycetoma is a chronic, granulomatous infection of the subcutaneous and cutaneous tissues that arises at the site of inoculation. The disease is characterized by swelling, with characteristic exudate draining to the skin surface through

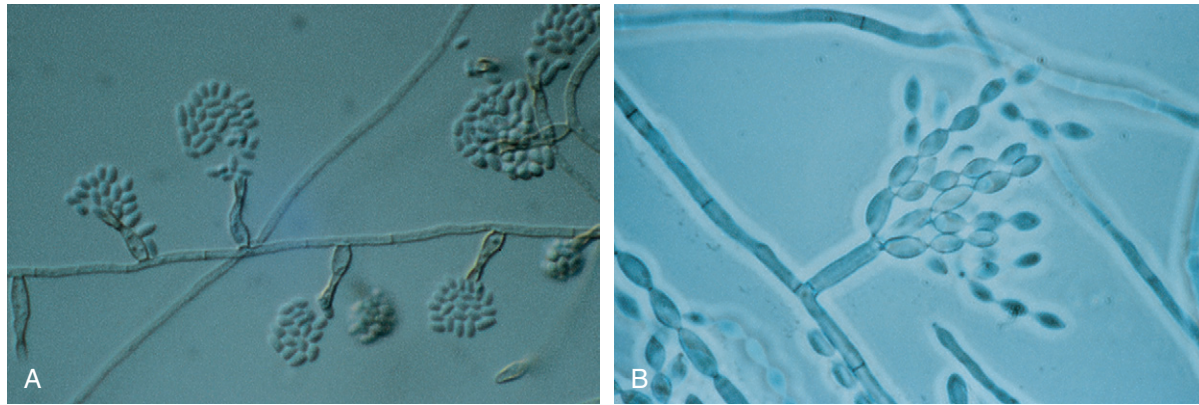


Fig. 27.18 **A**, Conidia of *Phialophora verrucosa* at the tips of phialides with collarettes (Nomarski optics, $\times 1000$). **B**, Conidial arrangement of *Cladophialophora carrionii* (lactophenol cotton blue preparation, $\times 1250$). (Courtesy Dr. Michael McGinnis.)

Table 27.3 Description of granules seen in eumycotic mycetomas

Fungus	Color	Size (mm)	Texture
<i>Scedosporium boydii</i>	White	0.5–1.0	Soft
<i>Fusarium falciforme</i>	White	0.2–0.5	Soft
<i>Madurella mycetomatis</i>	Black	0.5–5.0	Hard
<i>Trematosphaeria</i> (<i>Madurella</i>) <i>grisea</i>	Black	0.3–0.6	Soft
<i>Exophiala</i> spp.	Black	0.2–0.3	Soft

sinus tracts. Mycetomas can be caused by fungi or bacteria. Those caused by bacteria are referred to as *actinomycotic mycetomas*, and those caused by fungal agents are referred to as *eumycotic mycetomas*. Although clinical manifestations are similar for both types, the cause must be determined because the therapy for each is different.

Mycetomas occur primarily in tropical and subtropical areas but are also seen in temperate zones. The disease is endemic in India, Africa, and South America. Although mycetoma is an uncommon mycosis in the United States, the following species (in order of occurrence) are the most commonly incriminated agents: *Scedosporium boydii*, *Fusarium falciforme*, *Madurella mycetomatis*, *Trematosphaeria grisea* (formerly *Madurella grisea*), and *Exophiala* spp. Direct microscopic examination of the granules collected from the draining lesion immediately differentiates eumycotic from actinomycotic mycetomas (Table 27.3). Fig. 27.19A shows the branching filamentous rods of actinomycetes in contrast with the hyphal elements seen in eumycotic infections (see Fig. 27.19B).

Scedosporium boydii

Previously, it was thought that *Scedosporium apiospermum* was the anamorphic state of *S. boydii*, but molecular characterization has revealed that *S. boydii* and *S. apiospermum* are different species. *Pseudoallescheria boydii*, the teleomorph, and the anamorph, *S. boydii*, are the same species. Similarly, *P. apiosperma* (teleomorph) and its anamorph *S. apiospermum* are the same species. *S. boydii* produces oval conidia singly at the tips of conidiogenous cells (cells that make conidia) known as *annellides* (Fig. 27.20). The teleomorph form is noted by the

formation of cleistothecia containing ascospores (Fig. 27.21). This phenomenon occurs in fungi that are homothallic (ability of a single organism to undergo sexual reproduction without a mate). This isolate grows rapidly and produces colonies that are white to dark gray on potato dextrose agar at 22° and 35°C. Opinions regarding this fungus differ in relation to it being hyaline or phaeoid.

Fusarium falciforme

Fusarium falciforme produces mucoid clusters of single- or two-celled, slightly curved conidia borne from phialides at the tips of long, unbranched, multiseptate conidiophores. Conidia are held together in mucoid clusters at the apices of the phialides. This isolate is a hyaline, septate, filamentous mold. Colonies grow slowly and are grayish brown, becoming grayish violet.

Madurella

Madurella mycetomatis causes most cases of eumycotic mycetoma. *Madurella* spp. are phaeoid, septate fungi. Approximately 50% of the isolates of *M. mycetomatis* produce conidia from the tips of phialides, but many remain sterile. This species grows very slowly but is initially white, and becomes yellow, olivaceous, or brown, with a characteristic diffusible brown pigment with age. It grows best at 37°C, with slower growth at 40°C. DNA analysis led to the description of a new species, *Trematosphaeria grisea*, formerly named *M. grisea*.

Subcutaneous phaeohyphomycosis

Phaeohyphomycosis is a mycotic disease caused by darkly pigmented (phaeoid or dematiaceous) fungi. The term phaeohyphomycosis was coined to distinguish several clinical infections caused by phaeoid fungi from those distinct clinical entities known as *chromoblastomycosis*. In tissue, these fungi may form yeastlike cells that are solitary or in short chains or hyphae that are septate, branched, or unbranched and often swollen to toruloid (irregular or beaded). The agents responsible for these mycoses are organisms commonly found in nature, encompassing many genera of Hyphomycetes, Coelomycetes, and Ascomycetes. Fungi that appear to be regularly associated with this condition include *Exophiala* spp., such as *Exophiala dermatitidis*. A more complete list of genera

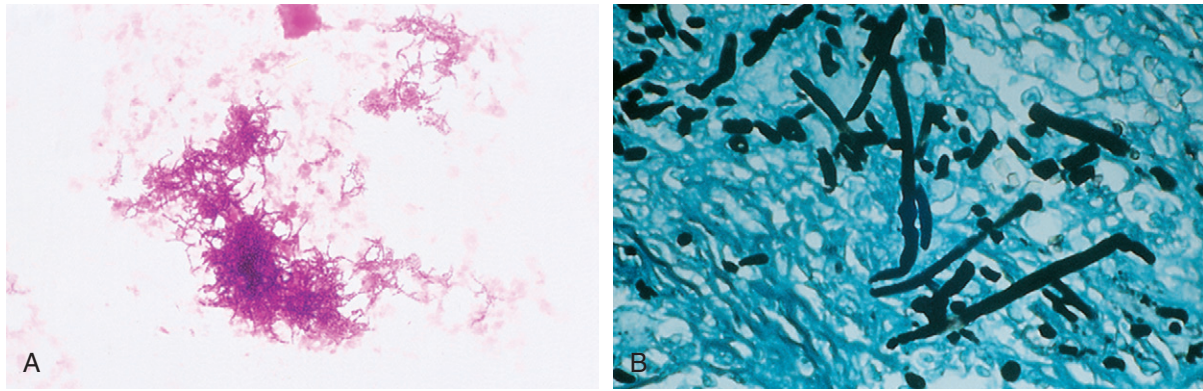


Fig. 27.19 Actinomycotic mycetoma showing fine-branching, filamentous rods in tissue sample (A), compared with the hyphal elements (hematoxylin and eosin stain $\times 1000$) (B) seen in eumycotic infections (Grocott-Gomori methenamine silver stain, $\times 1000$).

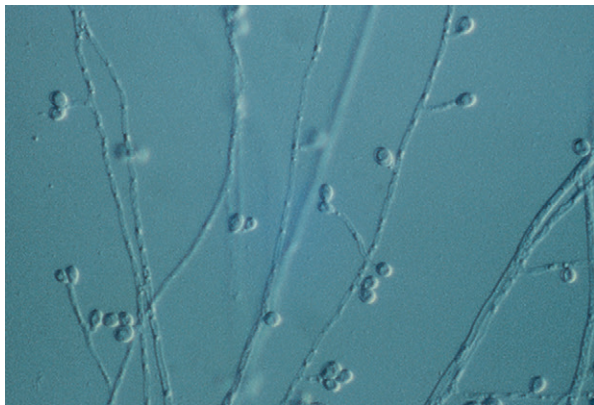


Fig. 27.20 *Scedosporium boydii* (Nomarski optics, $\times 625$).

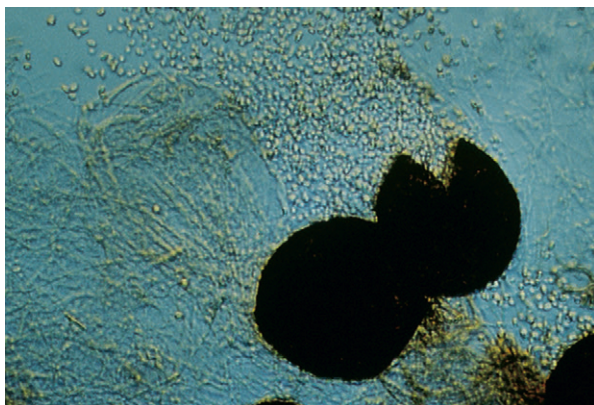


Fig. 27.21 Sexual structures (cleistothecia containing ascospores) of *Scedosporium boydii* (teleomorph *Pseudallescheria boydii*) (Nomarski optics, $\times 325$).

associated with subcutaneous phaeohyphomycosis is given in Box 27.1.

With most *Exophiala* spp., conidia are borne from annellides, with conidia aggregating in masses at the tips of the conidiophore, as seen in Fig. 27.22. *E. dermatitidis*, however, forms conidia at the tips of phialides (Fig. 27.23). This group of fungi produces olivaceous to black colonies that are initially yeastlike but become velvety at maturity.

BOX 27.1 Phaeoid genera inciting subcutaneous phaeohyphomycosis^a

<i>Alternaria</i>	<i>Mycocentrospora</i>
<i>Bipolaris</i>	<i>Ochroconis/Verruconis</i>
<i>Chaetomium</i>	<i>Oidiodendron</i>
<i>Cladosporium</i>	<i>Phaeosclera</i>
<i>Curvularia</i>	<i>Phialophora</i>
<i>Dactylaria</i>	<i>Phoma</i>
<i>Exophiala</i>	<i>Xylohypha</i>
<i>Fonsecaea</i>	

^aThis list is not all-inclusive.

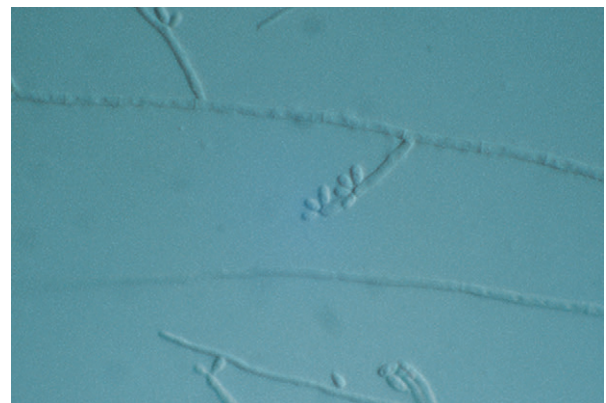


Fig. 27.22 Conidia of *Exophiala* sp. borne at the tips of annellides (Nomarski optics, $\times 1250$).

Sporothrix schenckii species complex

Clinical manifestations

The most commonly seen presentation of *Sporothrix schenckii* species complex infection is lymphocutaneous sporotrichosis. This chronic infection is characterized by nodular and ulcerative lesions along the lymph channels that drain the primary site of inoculation. Less commonly seen disease states include

fixed cutaneous sporotrichosis, in which the infection is confined to the site of inoculation and mucocutaneous sporotrichosis, a relatively rare condition. Primary and secondary pulmonary sporotrichosis and extracutaneous and disseminated forms of the disease may also occur.

Members of this complex, which include *Sporothrix schenckii*, *S. mexicana*, *S. globosa*, *S. luriei*, *S. albicans*, *S. inflata*, and *S. brasiliensis*, are commonly recovered from soil and are associated with decaying vegetation. Some are distributed worldwide, especially in warm, arid areas, such as Mexico, and also in moist, humid regions, such as Brazil, Uruguay, and South Africa. In temperate countries, such as France, Canada, and the United States, most cases of sporotrichosis are associated with gardening, particularly with exposure to rose thorns (rose handler's disease) and sphagnum moss.

Laboratory diagnosis

Direct examination of tissue might reveal members of the *S. schenckii* species complex as small, cigar-shaped yeasts (Fig. 27.24A). Although the organism is occasionally seen in a Gram-stained smear, wet mounts of material are often unrewarding because of the small numbers of organisms present. Microscopic examination from culture reveals thin, delicate hyphae bearing conidia developing in a rosette pattern at the ends of delicate

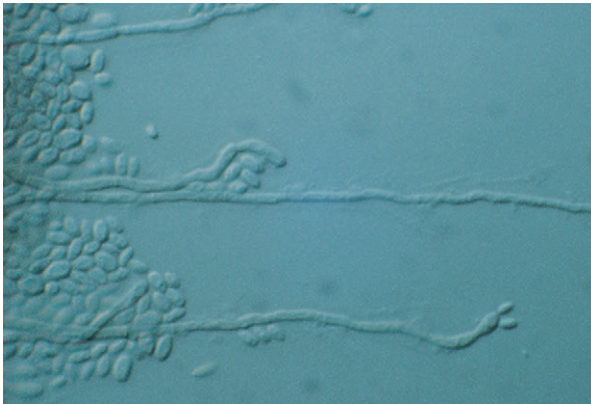


Fig. 27.23 Conidia of *Exophiala dermatitidis* borne at the tips of phialides as well as the black yeast synanamorph (Nomarski optics, $\times 1250$).

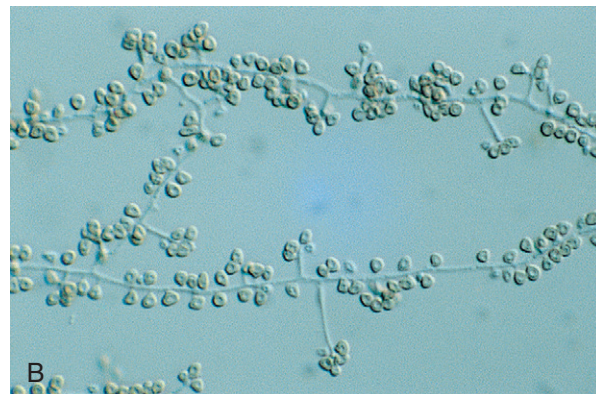
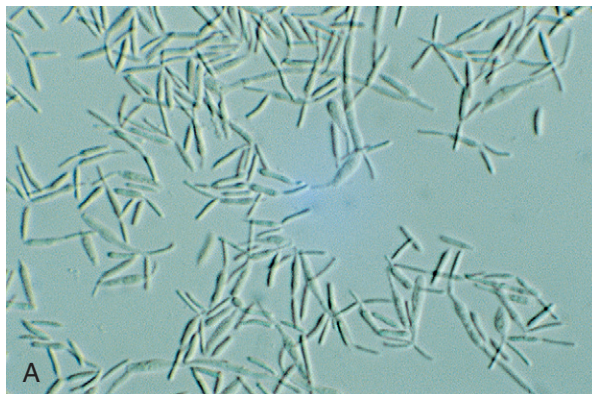


Fig. 27.24 **A**, Yeast phase of *Sporothrix schenckii* showing cigar-shaped yeast cells typical of the species (Nomarski optics, $\times 625$). **B**, Mold phase of *S. schenckii* revealing hyaline conidia borne at the ends of conidiophore in "rosettes" as well as phaeoid conidia along the sides of the hyphae (Nomarski optics, $\times 625$).

conidiophores. Dark-walled conidia are also produced along the sides of the hyphae and may be more readily viewable than the rosettes in mature cultures (see Fig. 27.24B).

Because members of this complex are dimorphic, cultures are examined at 22° and 37°C. These fungi grow well on most culture media, including those containing cycloheximide. The colony morphology at 22°C can be variable. At this temperature, colonies are often initially white, glabrous, and yeastlike, turning darker and more mycelial as they mature. Demonstration of dimorphism is important for the identification of these species. To induce mycelia conversion to yeast, the fungus is inoculated onto brain-heart infusion (BHI) agar supplemented with sheep red blood cells and incubated at 37°C in a CO₂ incubator. The formation of yeast colonies may require several subcultures. Complete conversion seldom occurs, but a portion of the colony will develop yeastlike cells.

Agents of systemic mycoses

Organisms that cause classic systemic fungal diseases have historically been categorized together because they share several characteristics, such as mode of transmission, dimorphism, and systemic dissemination. Although the term *systemic* generally refers to the organisms described here, it must be understood that any fungus, in an immunocompromised host, has the potential to become invasive and disseminate to sites far removed from the portal of entry.

The morphology of the systemic dimorphic at 22°C is described in Table 27.4. Conversion to the yeast or spherule form occurs when it is incubated at 35° to 37°C on enriched media with increased concentration of CO₂ (Table 27.5). Each agent has a fairly well-defined endemic area. The diseases are contracted generally by the inhalation of infectious conidia. Table 27.6 summarizes systemic mycoses, their agents, and their characteristics. All laboratory procedures to recover and identify these agents must be performed in a biological safety cabinet.

Blastomyces

Clinical manifestations

Blastomycosis is most prevalent in middle-age men, as are other systemic mycoses, presumably because occupational and recreational exposure to soil is often greater among men.

Table 27.4 Morphology of systemic fungi^a

Fungus	Macroscopic morphology	Microscopic morphology
<i>Blastomyces dermatitidis</i> , <i>B. gilchristii</i>	Slow to moderate growth White to dark tan Young colonies tenacious, older colonies glabrous to woolly	Oval, pyriform to globose smooth conidia borne on short, lateral hyphalike conidiophores
<i>Histoplasma capsulatum</i> , <i>H. mississippiense</i> , and <i>H. ohioense</i>	Slow growth White to dark tan with age Woolly, cottony, or granular	Microconidia small, one-celled, round, smooth (2–5 μm) Tuberculated macroconidia large, round (7–12 μm) Hyphalike conidiophores
<i>Coccidioides immitis</i> , <i>Coccidioides posadasii</i>	Rapid growth White to tan to dark gray Young colonies tenacious, older colonies cottony Tend to grow in concentric rings	Alternating one-celled, “barrel-shaped” arthroconidia with disjunct cells
<i>Paracoccidioides</i> spp.	Slow growth White to beige Colony glabrous, leathery, flat to wrinkled, folded or velvety	Colonies frequently only produce sterile hyphae Fresh isolates may produce conidia similar to those of <i>B. dermatitidis</i>

^aAt 22°C.Table 27.5 Mold to yeast conversion of thermally dimorphic fungi^a

Fungus	Culture media and temperature	Yeast form
<i>Blastomyces dermatitidis</i> , <i>B. gilchristii</i>	Blood agar, 37°C	Large yeast (8–12 μm) Blastoconidia attached by broad base
<i>Histoplasma capsulatum</i> , <i>H. mississippiense</i> , and <i>H. ohioense</i>	Pines medium, glucose-cysteine-blood, or BHI agar–blood agar, 37°C	Small, oval yeast (2–5 μm)
<i>Paracoccidioides</i> spp.	BHI agar–blood agar, 37°C	Multiple blastoconidia budding from single, large yeast (15–30 μm)

BHI, Brain-heart infusion.

^a*Coccidioides immitis* and *C. posadasii* can be converted to the spherule phase in modified Converse medium at 40°C in 5% to 10% carbon dioxide. Molecular assays are preferred to this procedure in the routine clinical laboratory.

Table 27.6 Summary of systemic mycoses

Fungus	Ecology	Clinical disease	Tissue form
<i>Blastomyces dermatitidis</i> , <i>B. gilchristii</i>	Mississippi and Ohio River valleys	Primary lung Chronic skin, bone Systemic, multiorgan	Large yeast (8–12 μm) Broad-based bud
<i>Histoplasma capsulatum</i> , <i>H. mississippiense</i> , and <i>H. ohioense</i> ^a	Ohio, Missouri, and Mississippi River valleys Bird and bat guano Alkaline soil	Primary lung Asymptomatic Immunodeficient hosts prone to disseminated disease	Small, oval yeast (2–5 μm) in histiocytes, phagocytes
<i>Coccidioides immitis</i> , <i>C. posadasii</i>	Semi-arid regions—southwest United States, Mexico, Central and South America In soil	Primary lung Asymptomatic Secondary cavity Progressive pulmonary Multisystem	Spherules (30–60 μm) contain- ing endospores
<i>Paracoccidioides</i> spp.	Central and South America In soil	Primary lung Granulomatous Ulcerative nasal and buccal lesions Lymph node involvement Adrenal glands	Thick-walled yeasts (15–30 μm) Multiple buds, “mariner’s wheel”

^a*Histoplasma duboisii* is endemic in Central Africa, and *H. suramericanum* is found in South America.

Although patients with primary infection may exhibit flulike symptoms, most are asymptomatic and cannot accurately define the time of onset. When the primary disease fails to resolve, pulmonary disease may ensue, with cough, weight loss, chest pain, and fever. Progressive pulmonary or invasive disease may follow, resulting in ulcerative lesions of skin and bone. In the immunocompromised patient, multiple organ systems may be involved, and the course may be rapidly fatal.

Blastomycosis is also known as *Gilchrist disease*, *North American blastomycosis*, and *Chicago disease*. It occurs primarily in North America and parts of Africa. In the United States, *B. dermatitidis* is endemic in the Mississippi and Ohio river basins, while the recently described *B. helicus* is found primarily in the west. Sporadic point source outbreaks have also occurred in the St. Lawrence River basin. The natural reservoir has not been unequivocally established, although the organism has been recovered from soil and from some natural environments. Apparently, only a very narrow range of conditions supports its growth. In areas in which the organism appears endemic, natural disease occurs in dogs and horses, with the disease process mimicking that seen in human infections.

Several species of *Blastomyces* are now recognized, including *B. dermatitidis*, *B. gilchristii*, and *B. helicus*, among others. Morphologically, *B. dermatitidis* and *B. gilchristii* are indistinguishable. *B. gilchristii* can be identified by using the sequences of the internal transcribed spacer 2 of the nuclear ribosomal ribonucleic acid (RNA) gene. The teleomorph or sexual form of *B. dermatitidis* is named *Ajellomyces dermatitidis*. It occurs only in rigidly controlled environments by mating of isolates with tester strains to produce gymnothecia containing ascospores. This teleomorph does not occur in the routine laboratory because the species is heterothallic, requiring two mating strains to produce the sexual form.

Laboratory diagnosis

For *B. dermatitidis* and *B. gilchristii*, examination of tissue or purulent material in cutaneous skin lesions may reveal large, spherical, refractile yeast cells, 8 to 15 μm in diameter, with a double-contoured wall and buds connected by a broad base (Fig. 27.25). KOH (10%) or calcofluor white (a fluorescent dye) may be used to enhance detection of the yeast cells (Fig. 27.26). In the mold phase, conidia are borne on short lateral branches that are ovoid to dumbbell-shaped and

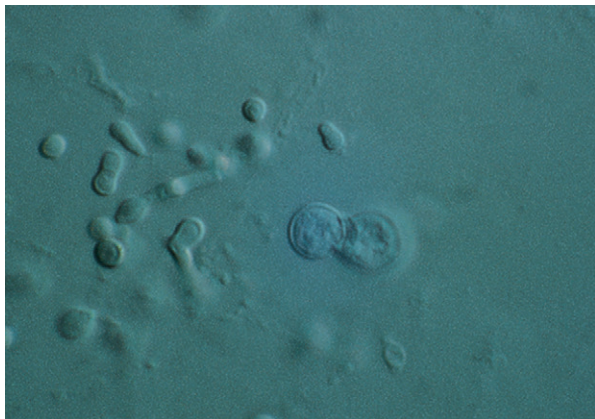


Fig. 27.25 Conversion of the mold phase of *Blastomyces dermatitidis* to the broad-based bud yeast form (Nomarski optics, $\times 1250$).

range in diameter from 2 to 10 μm . Because they resemble a variety of other fungi, the microconidia are not diagnostic (Fig. 27.27). An enzyme immunoassay (EIA) performed on serum and blood has a sensitivity of about 89%, but the specificity is low (79%).

In culture at 22°C, the organism can produce a variety of colony morphologies—white, tan, or brown—and may be fluffy to glabrous. Frequently, raised areas, termed *spicules*, are seen in the centers of the colonies. When grown at 37°C on suitable media, *B. dermatitidis* and *B. gilchristii* produce characteristic, broad-based, budding yeast cells. The mycelial phase of the systemic dimorphic fungi—*B. dermatitidis*, *B. gilchristii*, *Coccidioides immitis*, *Coccidioides posadasii*, *Histoplasma* spp., and *Paracoccidioides* spp.—requires confirmatory identification, typically by DNA probe and DNA sequencing. Because of low sensitivity and specificity, antigen detection methods are generally not used.

Coccidioides species

Clinical manifestations

Two very similar species that infect humans are *C. immitis* and *C. posadasii*. The inhalation of only a few arthroconidia produces primary coccidioidomycosis. Clinical infections

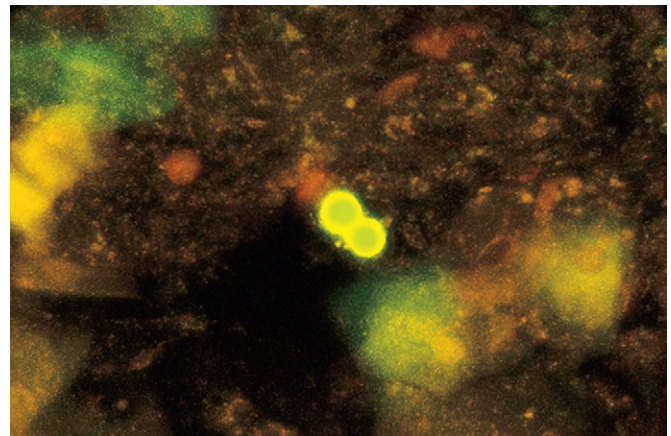


Fig. 27.26 Exsiccated sputum, smear, calcofluor white stain, fluorescence microscopy. Yeast (8 to 20 μm), round, thick-walled, broad-based bud. Morphology suggests *Blastomyces dermatitidis* ($\times 400$).

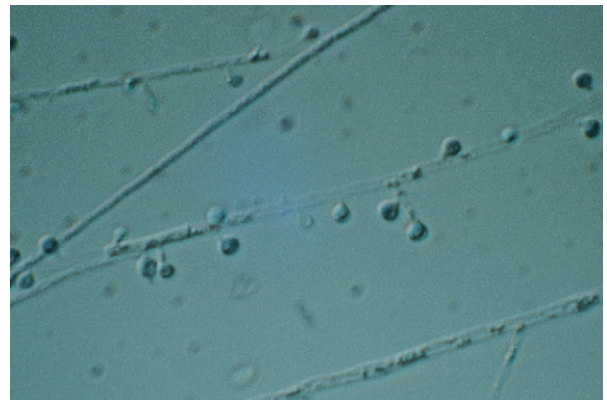


Fig. 27.27 Mold phase of *Blastomyces dermatitidis* grown on potato flakes agar (Nomarski optics, $\times 1250$).

include asymptomatic pulmonary disease and allergic manifestations. Allergy can manifest itself as toxic erythema, erythema nodosum (desert bumps), erythema multiforme (valley fever), and arthritis (desert rheumatism). Primary disease usually resolves without therapy and confers a strong, specific immunity to reinfection.

In symptomatic patients, fever, respiratory distress, cough, anorexia, headache, malaise, and myalgia can be present for 6 weeks or longer. The disease might then progress to secondary coccidioidomycosis, which can include nodules, cavity lung disease, and/or progressive pulmonary disease. Fewer than 3% of infected individuals develop systemic disease. Filipinos and Blacks run the highest risk of dissemination, with meningeal involvement being a common result of disseminated disease. The sex distribution ratio for clinically apparent disease has been reported to favor males. The exception is in pregnant women, in whom the dissemination rate equals or exceeds that for men.

Coccidioides spp. reside in a narrow ecologic niche known as the *Lower Sonoran life zone*, which is characterized by low rainfall and semi-arid conditions. Highly endemic areas include the San Joaquin Valley of California, the Maricopa and Pima counties of Arizona, and southwestern Texas. Outside the United States, endemic areas are found in northern Mexico, Guatemala, Honduras, Venezuela, Paraguay, Argentina, and Columbia. Because they are morphologically identical, molecular evaluation is required to differentiate *C. immitis* from *C. posadasii*. It appears that the species can be traced to specific geographic locations. *C. immitis* is encountered in the San Joaquin Valley region of California, whereas *C. posadasii* is found in the desert areas of the southwest United States, Mexico, and South America.

Laboratory diagnosis

After inhalation, the barrel-shaped arthroconidia, which measure 2.5 to 4 μm $\times$ 3 to 6 μm , round up as they convert to spherules. At maturity, the spherules (30 to 60 μm) produce endospores by a process known as *progressive cleavage*; rupture of the spherule wall releases the endospores into the bloodstream and surrounding tissues. These endospores, in turn, form new spherules (Fig. 27.28). Direct smear examination of secretions may reveal the spherules containing the endospores. Caution must be exercised when diagnosis is made by histopathologic means only. Small, empty spherules may resemble the yeast cells of *B. dermatitidis*, and the endospores

can be confused with the cells of *Cryptococcus*, *Histoplasma*, and *Paracoccidioides*. Direct antigen detection methods are limited.

Microscopic examination of the culture shows fertile hyphae arising at right angles to the vegetative hyphae, producing alternating (separated by a disjunct cell) hyaline arthroconidia (Fig. 27.29). When released, conidia have an annular frill at both ends. As the culture ages, the vegetative hyphae also fragment into arthroconidia. Although *Coccidioides* spp. do not readily convert to the spherule stage at 37°C in the laboratory, they produce a variety of mold morphologies at 22°C. Initial growth, which occurs within 3 to 4 days, is white to gray, moist, and glabrous. Colonies rapidly develop abundant aerial mycelia, and the colony appears to enlarge in a circular bloom. Mature colonies usually turn tan to brown to lavender in color.

Histoplasma

Clinical manifestations

Histoplasmosis is acquired by inhalation of the microconidia of *Histoplasma* species. The microconidia are phagocytized by macrophages in the pulmonary parenchyma. In the host with intact immune defenses, the infection is limited and is usually asymptomatic, with the only sequelae being areas of calcification in the lungs, liver, and spleen. With heavy exposure, however, acute pulmonary disease can occur. In the mild form of the disease, viable organisms remain in the host, quiescent for years, and are the presumed source of reactivation in individuals with abrogated immune systems. In immunocompromised individuals, *Histoplasma* can cause a progressive and potentially fatal disseminated disease. Chronic pulmonary histoplasmosis in patients with chronic obstructive pulmonary disease can also occur. Other various manifestations of the disease include mediastinitis, pericarditis, and mucocutaneous lesions.

Different *Histoplasma* spp. cause histoplasmosis, also known as *reticuloendothelial cytomycosis*, *Cave disease*, *Spelunker disease*, and *Darling disease*. Histoplasmosis occurs worldwide. The highest endemicity in the United States is found in the Ohio, Missouri, and Mississippi River deltas. Species include *H. capsulatum*, *H. mississippiense*, and *H. ohioense*, while *H. suramericanum* is found in South America. These organisms reside in soil with a high nitrogen content, particularly in areas

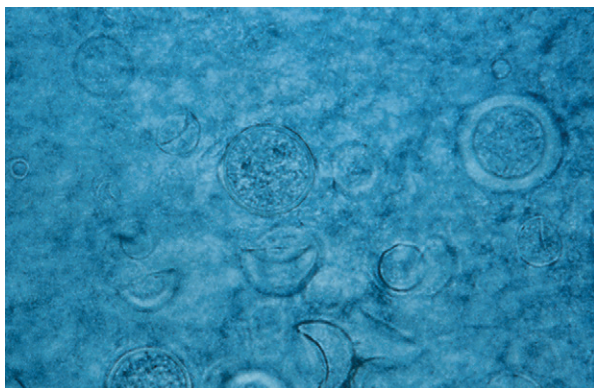


Fig. 27.28 Spherules of *Coccidioides immitis* in tissue ($\times 300$).

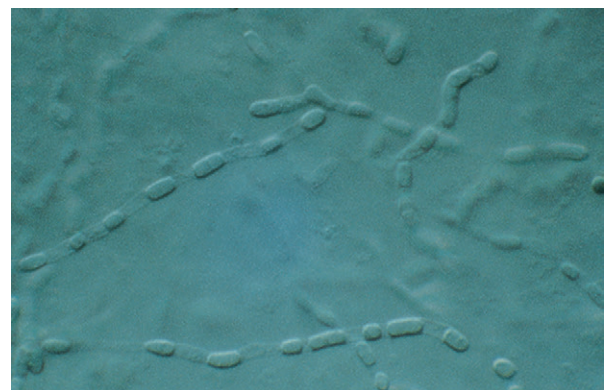


Fig. 27.29 Mold phase of *Coccidioides immitis*, 25°C (Nomarski optics, $\times 1250$).

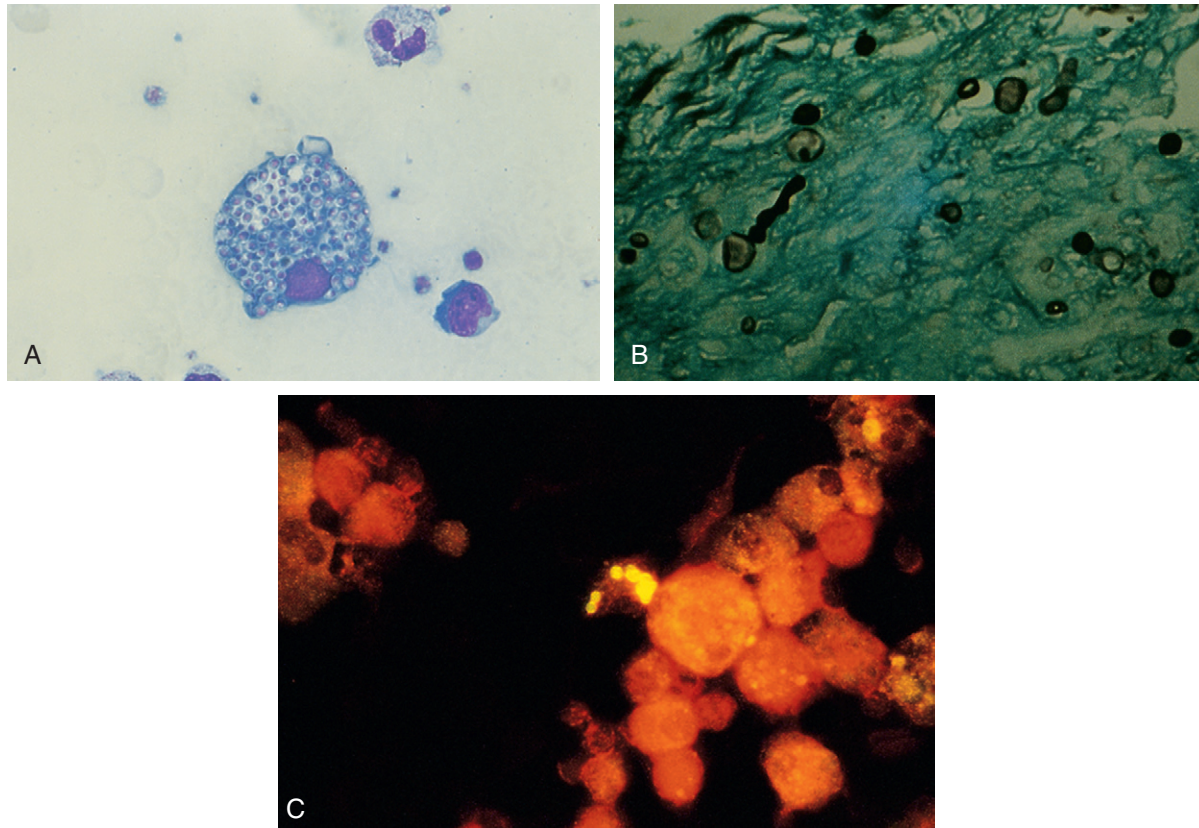


Fig. 27.30 **A**, Bone marrow aspirate stained with Giemsa stain showing the yeast cells of *Histoplasma capsulatum* inside the monocytes ($\times 1200$). **B**, Tissue phase of *H. capsulatum* (Gomori methylene stain, $\times 1200$). **C**, Bronchoalveolar lavage, cytocentrifuge preparation, calcofluor white stain, fluorescence microscopy. Fluorescent yeast (2 to $4\mu\text{m}$), small, budding. Morphology suggests *Histoplasma capsulatum* ($\times 1000$).

heavily contaminated with bat and bird guano. Skin testing previously demonstrated that about 80% of the population of long-term residents in endemic areas has been infected. *H. duboisii*, endemic in Central Africa, causes a clinically distinct form of disease involving primarily skin and bones, whereas *H. capsulatum* var. *farciminosum* causes epizootic lymphangitis in horses and mules.

Laboratory diagnosis

Careful examination of direct smear preparations of specimens for histoplasmosis frequently reveals the small yeast cells of *H. capsulatum* and similar *Histoplasma* species, particularly in infections seen in immunodeficient hosts. The yeast cells measure 2 to $3\mu\text{m}$ $\times$ 4 to $5\mu\text{m}$. When smears are stained with Giemsa or Wright stain, the yeast cells are commonly seen within monocytes and macrophages, occurring in significant numbers, as shown in Fig. 27.30A. The small cells, when found in tissue (see Fig. 27.30B), resemble the blastoconidia of *Candida glabrata*, but they can be differentiated by fluorescent antibody techniques or culture (see Fig. 27.30C).

Although microconidia can resemble *Chrysosporium* spp. and the macroconidia resemble *Sepedonium* spp., both saprobes do not produce two types of conidia in a single culture, nor are they dimorphic. Conversion of the mold form to the yeast form, using BHI agar incubated at 37°C , is confirmatory for *H. capsulatum*. Although complete conversion is seldom noted, a combination of both mycelial and yeast forms is sufficient for identification. *H. capsulatum* grows as a white-to-brownish mold. Early growth of the mycelial culture produces

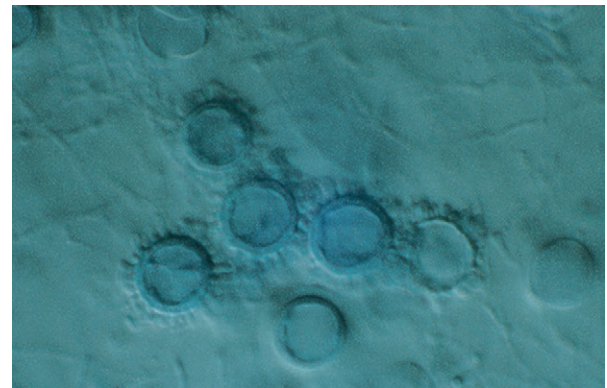


Fig. 27.31 Large tuberculate macroconidia of *Histoplasma capsulatum* (Nomarski optics, $\times 1250$).

round to pyriform microconidia measuring 2 to $5\mu\text{m}$. As the colony matures, large echinulate or tuberculate macroconidia characteristic for the species are formed (Fig. 27.31).

Direct antigen detection and serologic procedures for the diagnosis of histoplasmosis might be adjuncts to culture methods. Using EIA methods, *H. capsulatum* antigen can be detected from serum, CSF, and urine with a sensitivity of about 95%. Other assays include complement fixation, immunodiffusion, and latex agglutination to detect circulating antibody, along with fluorescent antibody test to detect viable or nonviable fungal elements in tissue sections. Currently,

the most useful test for rapid diagnosis of histoplasmosis is the combination of a DNA hybridization system and DNA sequencing. However, the cost of maintaining equipment for molecular procedures remains a challenge for regions with limited resources.

Paracoccidioides

Clinical manifestations

Although the primary route of infection for *Paracoccidioides* spp., which includes *P. brasiliensis*, *P. lutzii*, *P. americana*, *P. restrepiensis*, and *P. venezuelensis*, is pulmonary and infection is usually unapparent and asymptomatic, subsequent dissemination leads to formation of ulcerative granulomatous lesions of the buccal, nasal, and occasionally gastrointestinal mucosa. A concomitant striking lymph node involvement is also evident. Although *Paracoccidioides* spp. have a rather narrow range of temperature tolerance, as evidenced by its predilection for growth in cooler areas of the body (nasal and oropharyngeal), dissemination to other organs, particularly the adrenal glands, occurs with compromised host defenses. *Paracoccidioides* is the causative agent of paracoccidioidomycosis (South American blastomycosis, Brazilian blastomycosis, Lutz-Splendore-Almeida disease, paracoccidioidal granuloma), a chronic, progressive fungal disease endemic to Central and South America. Geographic areas of highest incidence are typically humid, high-rainfall areas, with acidic soil conditions.

Laboratory diagnosis

Direct microscopic examination of cutaneous and mucosal lesions demonstrates the characteristic yeast cells. The typical budding yeast measures 15 to 30 μm in diameter with multipolar budding at the periphery, resembling a mariner's wheel (Fig. 27.32). These daughter cells (2 to 5 μm) are connected by a narrow base, unlike the broad-based attachment in *Blastomyces dermatitidis*. Many buds of various sizes can occur, or there may be only a few buds, giving the appearance of a so-called *Mickey Mouse cap* to the yeast cell.

Paracoccidioides produces a variety of mold morphologies when grown at 22°C. Flat colonies are glabrous to leathery, wrinkled to folded, floccose to velvety, and pink to beige to brown with a yellowish-brown reverse, resembling those of *B. dermatitidis*. Microscopically, the mold form produces small (2 to 10 μm in diameter), one-celled conidia, generally

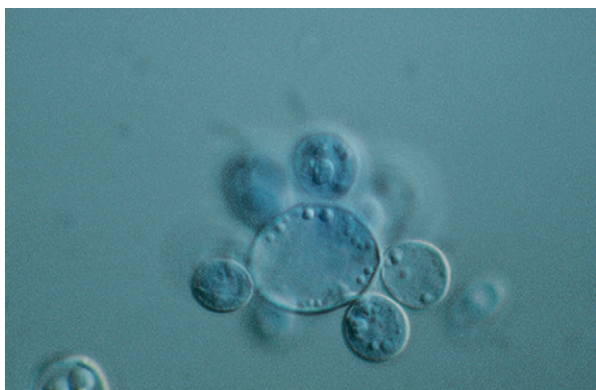


Fig. 27.32 Yeast phase ("mariner's wheel") of *Paracoccidioides brasiliensis* with multipolar budding (Nomarski optics, $\times 1250$).

indistinguishable from those observed with the mold phase of *B. dermatitidis* or the microconidia of *Histoplasma*. On BHI-blood agar, at 37°C, the mycelial phase rapidly converts to the yeast phase.

Talaromyces marneffei

Clinical manifestations

Talaromyces (formerly *Penicillium*) *marneffei* is unique among the *Talaromyces* spp., being dimorphic. *T. marneffei* is a common cause of systemic infection in immunocompromised patients who have visited the endemic region of Southeast Asia. This includes patients with AIDS, hematologic malignancies, or autoimmune disease and patients undergoing organ transplantations. Infections are usually disseminated, with multiple organ involvement. The fungus can be isolated from cutaneous lesions, which are frequently present in infected individuals. Disseminated disease is typically fatal. Studies showed that the fatality rate was about 28% among patients who did not have AIDS and received appropriate antifungal therapy.

Laboratory diagnosis

The yeastlike cells of *T. marneffei* can be detected in Wright-stained smears from skin lesions or biopsy specimens. The cells of *T. marneffei* resemble those of *Histoplasma*. They are oval to cylindric, measuring 3 to 6 μm long, and they may have a cross-wall. The mold form has sparse green aerial and reddish-brown vegetative hyphae and produces a red diffusible pigment. Polymerase chain reaction (PCR) tests have been described for identification confirmation. Serologic assays have been shown to be important in early diagnosis; however, they are not commercially available.

Agents of opportunistic mycoses

The terms *saprobe* and *saprophyte* have been used to describe free-living microorganisms that are present in the environment but are not typically of concern with regard to human disease. The line between saprobic and pathogenic organisms is increasingly blurred. The major reason for this development is the growing number of persons with defects in their immune system. For several decades, medical science has made advances in life-sustaining and life-lengthening treatments. As a result, a serious side effect of procedures such as organ transplantation and cancer chemotherapy is the short- or long-term insult to the host defenses. The spread of AIDS has greatly magnified the problem. Persons with AIDS constitute the prime targets for infection by a wide variety of microorganisms, including the recognized pathogenic fungi and a growing list of fungi previously regarded as harmless.

The types of disease caused by these fungi are as varied as the species, sometimes more so, because a given fungus can have multiple clinical presentations. Surgical wounds are ideal points of inoculation, allowing saprobes to become opportunistic agents of disease. Skin and nail bed infections as well as severe respiratory infections can be caused by a variety of fungi in patients with AIDS. A discussion of the most common saprobes that have been associated with opportunistic infections follows. These fungi are found worldwide in the environment and are often associated with decaying vegetation.

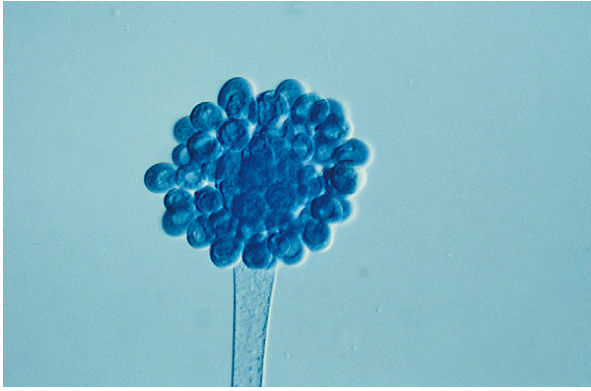


Fig. 27.33 *Cunninghamhamella* sp. (lactophenol cotton blue, $\times 450$).



Fig. 27.34 *Lichtheimia* sp. (Nomarski optics, $\times 450$).

Mucorales

Members of the order Mucorales are common environmental isolates associated with soil and plants. They contaminate grains, breads, and fruits and are most often associated with infections of the sinuses, lungs, and skin of immunocompromised patients. Studies have indicated an increased incidence of mucormycoses. Diabetes is a significant risk factor for these infections.

Cunninghamhamella

Cunninghamhamella spp. can be recovered from the sinuses or other organs during disseminated disease. Found worldwide, this isolate is common in the environment. Sporangioophores are erect, branching into several vesicles that bear sporangioles (Fig. 27.33), and may be covered with long, fine spines. These organisms are rapidly growing and form a cottony colony that is initially white but becomes gray with age.

Lichtheimia

Lichtheimia spp. have a predilection for vascular invasion, causing thrombosis and necrosis of the tissues. This agent, along with other fungi in the order Mucorales, is usually found in patients with poorly controlled diabetes. In this patient population, the infection usually begins in the sinuses, where conidia are inhaled and take up residence. From the sinuses, infection rapidly spreads to the orbits, face, palate, and brain. This presentation is known as **rhinocerebral mucormycosis**. Other sites of infection have been noted in patients with cancer, in whom cutaneous, subcutaneous, and systemic diseases occur. *Lichtheimia* spp. are found worldwide and are often associated with soil or decomposing organic matter.

Lichtheimia hyphae are broad and ribbonlike, with few septations (Fig. 27.34). Erect sporangioophores, solitary or in groups (slightly branched), terminate in an apophysis surrounded by a sporangium. Sporangiospores are smooth and ovoid. Internodal **rhizoids** (short, thin projections that anchor the growing cells to substratum) are present. Colonies are woolly and grow rapidly, completely covering the culture medium. Colony color is initially white, becoming gray to gray-brown with age.

Mucor

As with other Mucorales, *Mucor* spp. are associated with rhinocerebral mucormycosis in addition to disseminated disease. *Mucor* spp. are commonly isolated from the environment

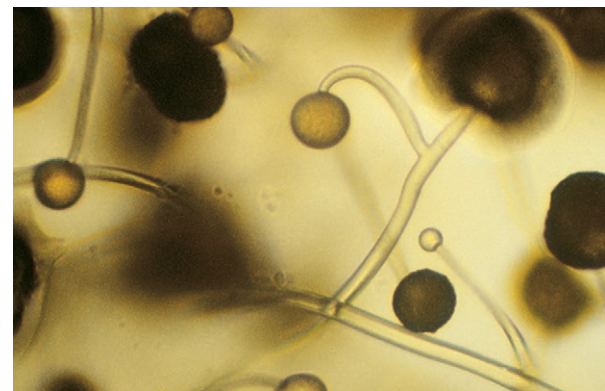


Fig. 27.35 *Mucor* sp. (unstained, $\times 450$).

worldwide. Sporangiospores are formed in sporangia on erect sporangioophores (Fig. 27.35). Rhizoids, typical of some Mucorales, are absent in most *Mucor* spp. The sporangia frequently remain intact, as opposed to *Rhizopus* spp., in which the sporangia typically collapse. *Mucor* spp. grow rapidly and form cottony, dirty white colonies that become mousy brown to gray with age.

Rhizopus

Rhizopus spp. are the most common Mucorales causing human disease. These are typically involved in patients with poorly controlled diabetes, presenting as rhinocerebral mucormycosis. *Rhizopus* spp. may be refractory to treatment and may be recovered from almost any source. With worldwide distribution, this isolate is easily recovered from the environment in decaying vegetation.

Rhizopus spp. are rapidly growing and have erect sporangioophores terminating in dark sporangia and sporangiospores (Fig. 27.36). At the base of the sporangioophores are brown rhizoids. Separate clusters of sporangioophores are joined by stolons, arching filaments that terminate at the rhizoids. The sporangia are typically fragile and are not easily retained when making slide culture preparations, resulting in an umbrella-shaped structure at the end of the conidiophores. *Rhizopus* spp. produce rapidly growing, woolly colonies that cover the entire surface of the culture medium. Colonies are initially white but become gray to brown with age.

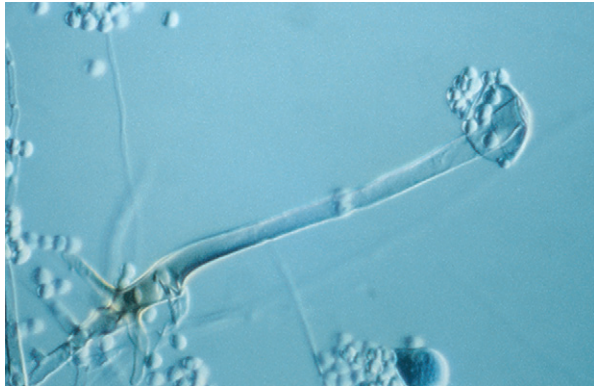


Fig. 27.36 *Rhizopus* sp. (Nomarski optics, $\times 450$).

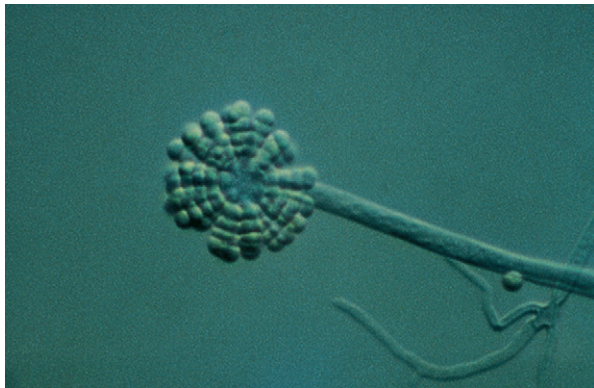


Fig. 27.37 *Syncephalastrum* sp. (Nomarski optics, $\times 450$).

Syncephalastrum

Syncephalastrum is rarely implicated in human disease but has been documented in cutaneous infections. This fungus is found in soil and decaying vegetation. Microscopically, erect sporangiophores are noted. Each sporangiophore has a large columella on which merosporangia, containing stacks of sporangiospores, are formed (Fig. 27.37). Isolates are sometimes confused with *Aspergillus* on initial examination. Colonies are rapidly growing and are initially white and become gray with age. The growth rate is rapid, with colonies covering the entire surface of the agar.

Septate and hyaline saprobes

Aspergillus

Aspergillus spp. are ubiquitous environmental saprobes and can frequently be isolated from a number of hospital sites, including ventilation systems and food. Aspergilli are the second most commonly isolated fungus after *Candida* spp. *A. fumigatus* is the species most often seen; other commonly isolated pathogenic species include *A. flavus*, *A. terreus*, and *A. niger*. Their conidia are constantly inhaled, but they are generally readily cleared in healthy, immunocompetent individuals. Mortality from infections caused by the aspergilli remains high, especially in the immunocompromised host.

Aspergillus spp. are the most frequent cause of disease in bone marrow transplant recipients, in addition to other

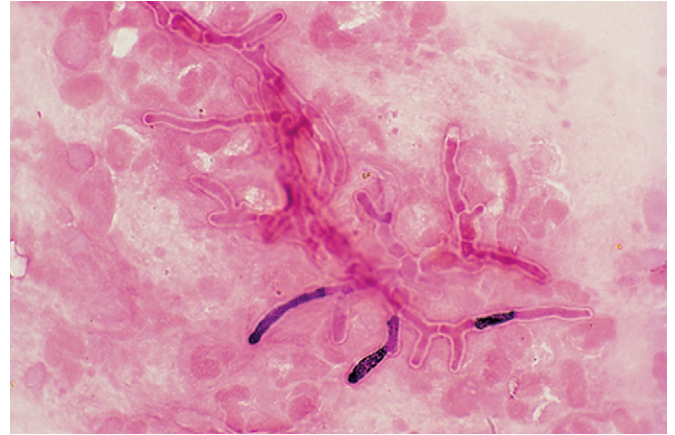


Fig. 27.38 Expectorated sputum, smear, Gram stain, light microscopy. Gram-variable hyphae present (3 to $10\mu\text{m}$), septate, branched 45-degree angle. Morphology suggests *Aspergillus* spp. ($\times 1000$).



Fig. 27.39 Expectorated sputum, smear, Gram stain, light microscopy. Gram-positive conidia (2 to $4\mu\text{m}$), in chain. Morphotype suggests *Aspergillus* spp. in cavity with air interface. Impression: cavitary aspergillosis ($\times 1000$). Care must be taken not to mistake these conidia for streptococci or for yeasts (see Fig. 27.40).

transplant recipients and those with cancer. Infection is initiated following inhalation of conidia. In the lung air spaces, conidia germinate into hyphae, which invade the tissue, including blood vessels (Figs. 27.38, 27.39, and 27.40). Fungal elements are easily seen with the fluorescent calcofluor white stain (Fig. 27.41). Although not all patients have chest pain, it is not uncommon for pneumonia-like symptoms to appear. The infection easily spreads hematogenously, and it is not uncommon to find multiorgan system involvement, including the brain, liver, heart, and bone (Fig. 27.42). *Aspergillus* spp. also trigger allergic reactions and are a common cause of sensitivities to molds. Another frequent presentation is that of so-called *fungus balls* in the lungs of agricultural workers who routinely are in contact with fungal conidia from environmental sources. Chronic pulmonary aspergillosis may occur in patients with structural damage to their lungs caused by other diseases, including tuberculosis and sarcoidosis.

Aspergilli may be uniseriate or biseriate. Uniseriate species are those whose phialides attach directly to the vesicle at the end of the conidiophore. Biseriate species possess

a supporting structure called a *metula*. Metulae attach directly to the vesicle, and attached to each of the metulae are phialides (Fig. 27.43). Conidia are produced from the phialides. Other characteristics include an erect conidiophore arising from a foot cell within the vegetative hyphae. It is also important to note whether or not phialoconidia remain in long chains or are easily disturbed into individual phialoconidia (see Figs. 27.39 and 27.40). Chains of conidia can be aligned in very straight, parallel columns or in a radiating pattern around the vesicle, and the conidia may be rough or smooth.

The color of *Aspergillus* spp. colonies is derived from conidia. Colors range from black to white and include yellow, brown, green, gray, pink, beige, and tan. Some species also form diffusible subsurface pigments on a variety of media. A granular texture is seen in species with abundant conidial formation. Most known pathogens in this group form green- to tan-colored colonies. Many species of aspergilli have been implicated in human disease. Although some are easily distinguished from others, molecular and proteomic tests aid in definitive identification at the species level.

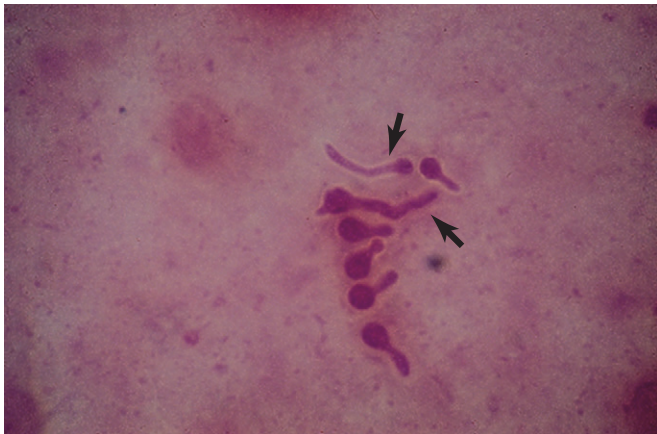


Fig. 27.40 Expectorated sputum, smear, calcofluor white stain, fluorescence microscopy, MPV. Fungal hyphae present (3 to 10 μm), septate, branched 45-degree angle. Morphology suggests *Aspergillus* spp. ($\times 400$).

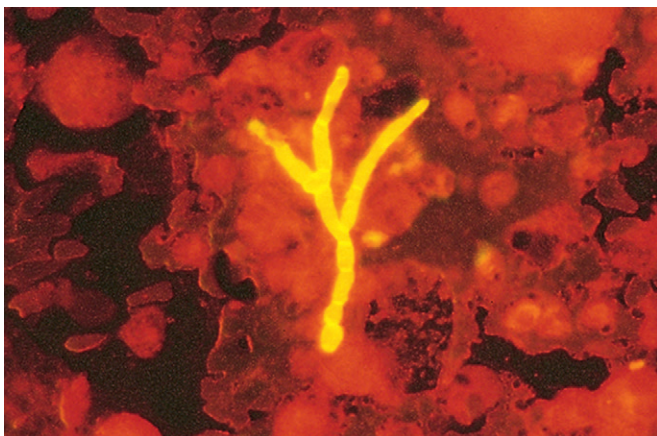


Fig. 27.41 Expectorated sputum, smear, Gram stain, light microscopy. Gram-negative conidia (2 to 4 μm), sporulating (arrows) ($\times 1000$). Care must be taken not to confuse these conidia with yeast germ tubes.

Beauveria

Beauveria bassiana is a rare human isolate, uncommonly associated with keratitis. This fungus is a known insect pathogen and is found worldwide on vegetation and in soil. Abundant, single-celled, tear-shaped sympoduloconidia are formed on sympodulae, which taper extremely from a rather swollen base (Fig. 27.44). Conidiophores may cluster in some isolates to form radial tufts. Colonies are hyaline, moderately rapidly growing, fluffy colonies, sometimes developing a powdery surface reminiscent of *T. mentagrophytes*.

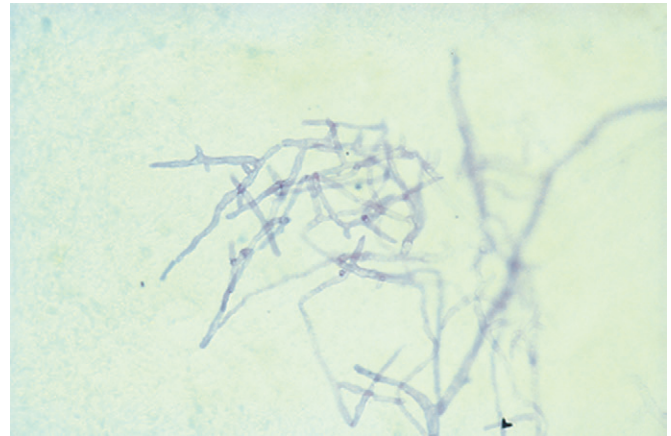


Fig. 27.42 Brain abscess, smear, toluidine blue stain, light microscopy. Fungal hyphae present (3 to 10 μm), septate, branched 45-degree angle. Morphology suggests *Aspergillus* spp. ($\times 400$). Impression: cerebral aspergillosis.



Fig. 27.43 *Aspergillus* sp. (Nomarski optics, $\times 450$).

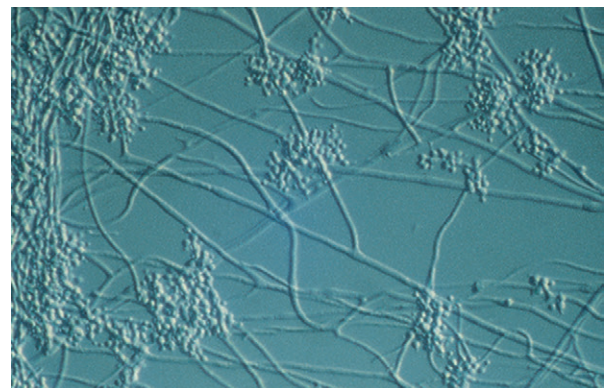


Fig. 27.44 *Beauveria* sp. (Nomarski optics, $\times 450$).

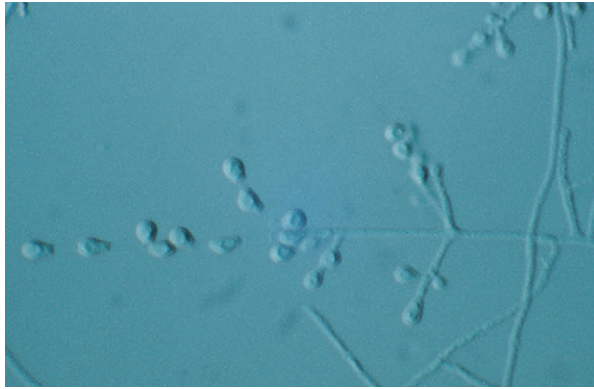


Fig. 27.45 *Chrysosporium* sp. (Nomarski optics, $\times 450$).

Chrysosporium

A rare cause of disease, *Chrysosporium* spp. have been recovered from nails and skin lesions. *Chrysosporium zonatum* has been linked to pneumonia and osteomyelitis in immunocompromised patients. These organisms are found in the environment worldwide. Microscopically simple, wide-based, single-celled conidia are produced on nonspecialized cells (Fig. 27.45). The conidiogenous cell disintegrates or breaks to release conidia. Both arthroconidia and aleurioconidia may be seen. Colonies are hyaline with a moderate growth rate and with age can develop light shades of pink, gray, or tan pigment.

Fusarium

Fusarium spp. are frequently seen in mycotic keratitis. In the United States, a multistate outbreak involving more than 100 individuals wearing soft contact lenses was reported in 2006. The outbreak was associated with a particular brand of contact lens solution and occurred typically in patients who wore lenses continuously without removal for several days at a time. From 2013 to 2014, seven cases of fungemia due to *Fusarium oxysporum*, related to central line catheters, were reported in a pediatric cancer center. In bone marrow transplant recipients with infections caused by fusaria, the mortality rate approaches 100%. Unless a patient regains some cell-mediated immunity, mortality is certain because of the ability of these fungi to grow and continue to invade despite antifungal therapy. Patients present with high fever, possibly disseminated skin lesions, and in some patients, fungemia. *Fusarium* spp. can be recovered in blood culture systems. Care should be taken when reviewing positive blood cultures because isolates typically appear yeastlike on initial recovery.

Normally, abundant macroconidia with fewer microconidia are produced on vegetative hyphae (Fig. 27.46). Macroconidia are banana or canoe shaped and are formed singly, in small clusters, or clustered together in mats termed *sporodochia*. Macroconidia typically are multicelled. *Fusarium* is a rapidly growing hyaline fungus that can develop various colors with age, ranging from rose to mauve to purple to yellow.

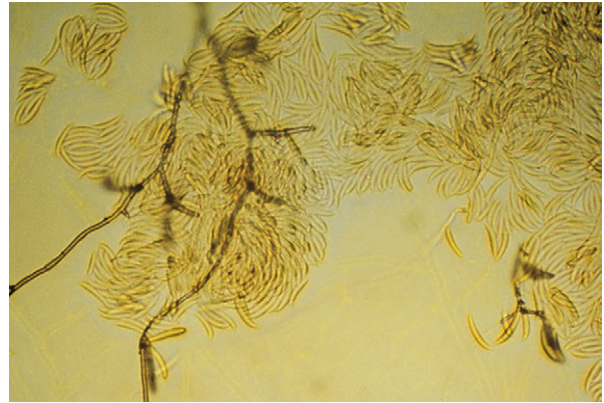


Fig. 27.46 *Fusarium* sp. (unstained, $\times 450$).



Fig. 27.47 *Geotrichum* sp. (Nomarski optics, $\times 650$).

Geotrichum

Geotrichum has been implicated in pulmonary disease in immunocompromised patients. Microscopic evaluation reveals abundant arthroconidia formed from vegetative hyphae that occur singly or may be branched (Fig. 27.47). Colonies appear white to cream and yeastlike and can be confused with *Trichosporon* spp. Occasionally, aerial mycelia form, producing colonies that resemble those of *Coccidioides* spp.

Purpureocillium

Purpureocillium lilacinum, previously *Paecilomyces lilacinus*, has been associated with cutaneous and subcutaneous infections, in addition to pyelonephritis, endocarditis, and pulmonary infections in immunocompromised and immunocompetent patients. It was recovered in a hospital outbreak with a high rate of associated death. Microscopically, care must be taken to avoid confusion between *Purpureocillium* and *Penicillium* spp. Phialides of *Purpureocillium* are generally longer and more obviously tapered, and they may be singly formed or arranged in a verticillate pattern, on which long chains of spindle-shaped or somewhat cylindric conidia are formed (Fig. 27.48).

Purpureocillium grows rapidly and usually form flat, granular to velvety colonies in shades of tan, brownish gold, or mauve. Green or blue-green colors are not seen. Infections with *Purpureocillium* are potentially serious and difficult to treat, as *P. lilacinum* is intrinsically resistant to amphotericin B and other polyenes; care should be taken when encountering fungi that are mauve in color. Molecular testing is recommended to get a definitive species identification.

Case check 27.1

Unlike most molds, *Fusarium* spp. are commonly recovered from blood. As demonstrated in the Case in Point, care must be taken for proper diagnosis because colonies may initially appear yeastlike on subculture but rapidly become woolly in appearance as hyphae are formed.

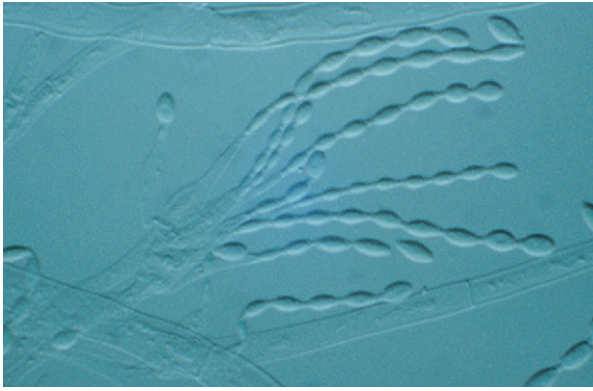


Fig. 27.48 *Purpureocillium lilacinum* (*Paecilomyces lilacinus*) (Nomarski optics, $\times 650$).

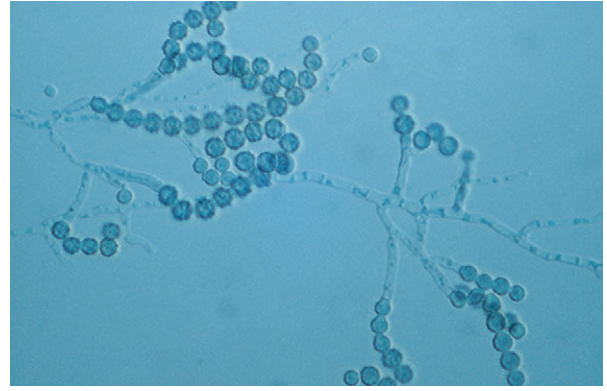


Fig. 27.50 *Scopulariopsis* sp. (lactophenol cotton blue, $\times 450$).



Fig. 27.49 *Penicillium* sp. (Nomarski optics, $\times 650$).

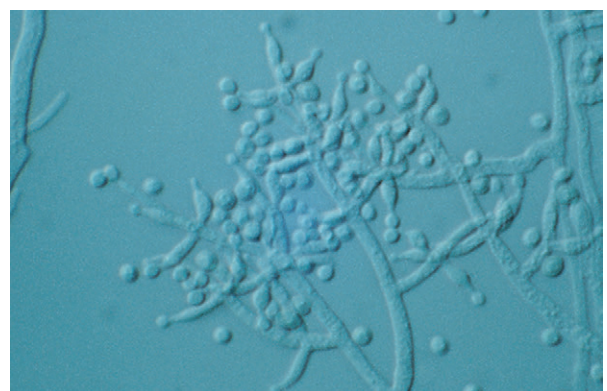


Fig. 27.51 *Trichoderma* sp. (Nomarski optics, $\times 650$).

Penicillium

Because many *Penicillium* spp. are inhibited at 37°C, they do not frequently cause infections. Most reports of disease involve chronic fungal sinusitis. Ubiquitous in nature, these fungi can be recovered from any location worldwide. Conidiophores are erect, sometimes branched, with metulae bearing one or several phialides on which oval to ovoid conidia are produced in long, loose chains (Fig. 27.49). This commonly seen fungus is a rapid grower, with colonies usually in shades of green or blue-green.

Scopulariopsis* and *Microascus

Scopulariopsis and *Microascus* spp. are commonly isolated from nail specimens and have been implicated in pulmonary disease in immunocompromised patients. These fungi are recovered from the environment worldwide. Conidiophores occur singularly or can be in clusters (Fig. 27.50). Conidia are formed from annellides, which increase in length as conidia are formed. The truncate-based conidia tend to remain in chains on the annellides. *Scopulariopsis* and *Microascus* spp. grow moderately rapidly and form colonies covered by tan-to-buff conidia. Some species are phaeoid.

Trichoderma

Trichoderma spp. are emerging as pathogens that can cause a range of infections, including pulmonary and skin infections, in the immunocompromised host. This isolate is readily recovered from the environment worldwide. *Trichoderma* spp.

are rapidly growing and form hyaline hyphae that give rise to yellow-green to green patches of conidia formed on clusters of tapering phialides (Fig. 27.51). Conidia may remain clustered in balls at the phialide tips. Mature colonies are intensely green and granular, with an abundance of conidia.

Septate and phaeoid saprobes

Alternaria

Based on phylogenetic studies and morphology, *Alternaria*, which consists of several saprophytic and pathogenic species, is currently grouped into 26 sections. Although *Alternaria* spp. can be recovered from almost any source, they are primarily implicated in chronic fungal sinusitis. The disease is often misdiagnosed, and patients are often treated for an extended period for bacterial sinusitis. The infection rarely spreads beyond the sinuses in immunocompetent hosts but can be found systemically in those with immune suppression. *Alternaria* spp. are found worldwide on grasses and leaves. They have been implicated in tomato rot and are readily recovered from the environment in air-settling plates.

Microscopic evaluation reveals short conidiophores bearing conidia in chains that lengthen in an acropetal fashion (Fig. 27.52). Multicelled conidia have angular cross-walls and taper toward the distal end. *Alternaria* spp. are phaeoid, rapidly growing fungi with colonies ranging from shades of gray to brown to black.

Aureobasidium

Infections by *Aureobasidium* spp. are rare but have been traced to contaminated dialysis lines, catheters, and similar devices. This organism may be recovered from blood, tissues, and abscesses. It is recovered worldwide primarily in wet environments, such as from shower tiles and water lines.

Microscopic examination reveals hyaline hyphae giving rise to hyaline conidia directly from the vegetative hyphae. With age, phaeoid hyphae develop and break up into arthroconidia, which do not bear hyaline conidia. These arthroconidia are responsible for the darkening colony morphology. *Aureobasidium* spp. grow moderately rapidly to significantly rapidly and have a yeastlike consistency. Young cultures are off-white to pink, but they become black with age, with the production of darkly pigmented arthroconidia.

Chaetomium

Infections by *Chaetomium* organisms have been reported in the brains of patients with central nervous system disease. Several of these patients have been identified as intravenous drug abusers. These fungi are found in the environment and have a predilection for cellulose products. They have been known to devastate printed literature and library holdings and have been associated with problems in indoor air quality.

Microscopically, numerous perithecia are typically seen (Fig. 27.53). These perithecia are pineapple shaped and are ornamented with straight or curled hairs or setae. The asci contained within the perithecia are evanescent, so at maturity, the pigmented, lemon-shaped ascospores are released within the

perithecium. Colonies are moderately rapid to rapidly growing and begin dirty gray and become phaeoid with age. Some species produce a diffusible pigment that turns the agar completely red.

Cladosporium

An infrequent cause of disease, *Cladosporium* spp. are primarily recovered as laboratory contaminants. Infections are typically confined to the sinuses or following traumatic inoculation. Ubiquitous in nature, this isolate can be recovered from almost any location in the world.

Cladosporium spp. form brown to olive to black hyphae and conidia (Fig. 27.54). Conidiophores are erect and can branch into several conidiogenous cells. Spherical to ovoid conidia form blastically on the end of each previously formed conidium. Branched conidium-bearing cells may dislodge, and the three scars on each of these cells give them the appearance of a shield. Generally, conidial chains of the saprobic species break up easily, whereas those of pathogenic species remain connected. These organisms are slowly to moderately growing phaeoid fungi, with granular velvety to fluffy colonies, ranging in color from olive to brown or black.

Curvularia

Curvularia spp. isolates are usually implicated in chronic sinusitis in immunocompetent patients. Found worldwide, this fungus is frequently recovered from grass, leaves, and decaying vegetation. Multicelled conidia are produced on sympodial conidiophores (Fig. 27.55). This genus is among



Fig. 27.52 *Alternaria* sp. (unstained, $\times 450$).

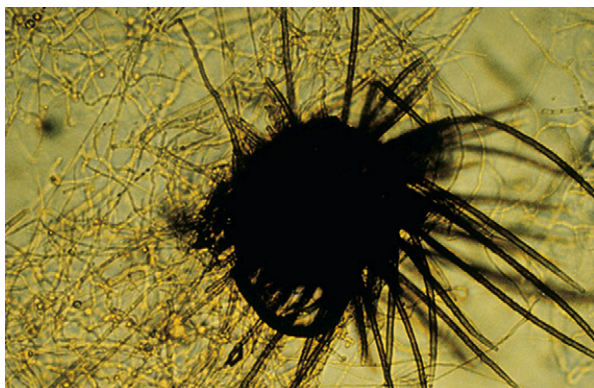


Fig. 27.53 *Chaetomium* sp. (unstained, $\times 650$).



Fig. 27.54 *Cladosporium* sp. (Nomarski optics, $\times 650$).

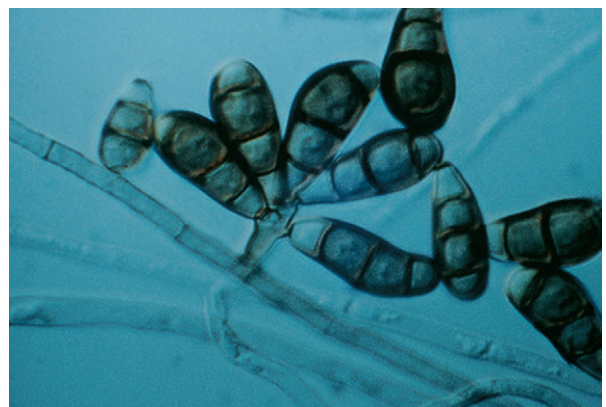


Fig. 27.55 *Curvularia* sp. (Nomarski optics, $\times 450$).

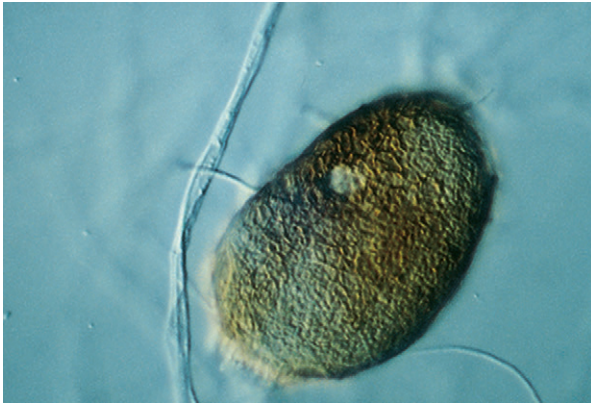


Fig. 27.56 *Phoma* sp. (Nomarski optics, $\times 650$).

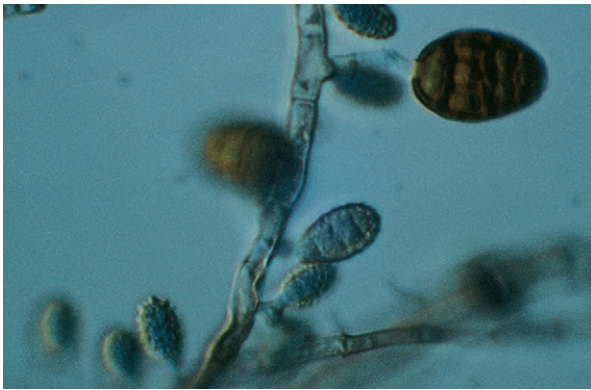


Fig. 27.57 *Pithomyces* sp. (Nomarski optics, $\times 450$).

the easiest to identify because of the frequently seen crescent-shaped conidia with three to five cells of unequal size and an enlarged central cell. Several species previously classified in the genus *Bipolaris* are now considered to be members of *Curvularia*. These fungi form a rapidly growing phaeoid colony that is cottony and dirty gray to black.

Phoma

Disease caused by *Phoma* spp. is usually secondary to traumatic inoculation. *Phoma* spp. produce pycnidia, which appear as black fruiting bodies that are globose and lined inside with short conidiophores (Fig. 27.56). Large numbers of hyaline conidia are generated in the pycnidium and flow out of a small apical pore. *Phoma* spp. produce a moderately rapid growing, gray-to-brown colony.

Pithomyces

Disease caused by *Pithomyces* spp. is usually secondary to traumatic inoculation. Conidia are somewhat barrel shaped, formed singly on simple short conidiophores (Fig. 27.57). Conidia have both transverse and longitudinal cross-walls and are often echinulate. *Pithomyces* spp. produce rapidly growing phaeoid colonies.

Ulocladium

Ulocladium spp. are now classified among the sections in *Alternaria*. These species are sometimes implicated in subcutaneous infections, usually following traumatic inoculation. Conidiophores bear dark, multicelled conidia on sympodial conidiophores (Fig. 27.58). Conidia have angular cross-walls

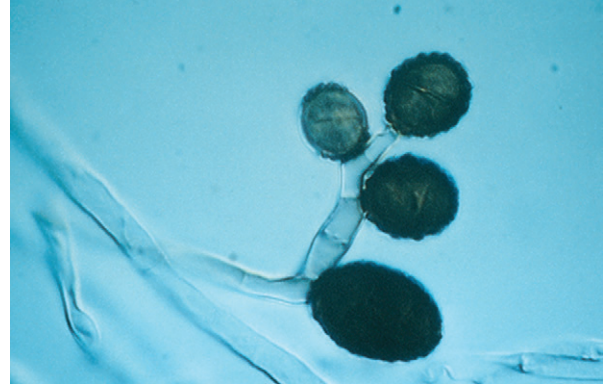


Fig. 27.58 *Ulocladium* (lactophenol cotton blue, $\times 450$).

and, in some species, echinulate surfaces. These species are rapidly growing phaeoid fungi, forming colonies ranging in color from brown to olivaceous to black.

Agents of yeast infections

The escalating incidence of yeast and yeastlike fungi isolated from patient specimens has emphasized the importance of identifying yeast isolates to the species level. With greater immunosuppression, the variety of organisms implicated in disease also expands. *Candida albicans* is the fourth most common cause of bloodborne infection in the United States, accounting for 10% to 15% of all hospital-acquired septicemia cases. Isolation of other yeasts from clinical samples is also increasing. Infections caused by many yeasts are extremely aggressive and difficult to treat.

Yeast can be classified into one of two groups: yeasts and yeastlike fungi. Isolates that reproduce sexually, by forming either ascospores or basidiospores, are truly yeasts. Most isolates that are not capable of sexual reproduction or whose sexual state has not yet been discovered are correctly termed *yeastlike fungi*. For ease of discussion, all isolates are referred to here as *yeasts*.

General characteristics

Molds and yeasts are very different morphologically, but some of the macroscopic characteristics used as aids in identifying molds can also be used to identify yeasts. Macroscopic characteristics include color and colony texture. The color of a yeast colony ranges from white to cream or tan, with a few species forming pink- to salmon-colored colonies. Some yeast isolates, referred to as *phaeoid yeasts*, are darkly pigmented because of melanin in their cell walls. Phaeoid yeasts are associated with several species of the polymorphic fungi and are discussed elsewhere in this chapter.

The texture of the yeast colonies also differs. For example, *Cryptococcus* spp. tend to be mucoid and can flow across the plate, a trait shared by some bacteria, such as *Klebsiella* spp. Some yeasts are butterlike, and others range in texture from velvety to wrinkled. Strain-to-strain variation in texture may be noted within a species.

Candida

Candida spp. are the most notorious agents of yeast infection. Clinical disease ranges from superficial skin infections

to disseminated disease. *C. albicans* is a major cause of yeast infection in the world. It is recovered as normal biota from a variety of sites, including skin, the oral mucosa, the digestive tract, and the vagina. When host conditions are altered, however, this organism is capable of causing disease in almost any site. In individuals with an intact immune system, infections are localized and limited. One of the most widely recognized manifestations of *C. albicans* infection is thrush (oropharyngeal candidiasis), an infection of the oral mucosa. **Thrush** is also recognized as an indicator of immunosuppression. Among individuals infected with human immunodeficiency virus (HIV) and those receiving prolonged antibacterial therapy or other chemotherapeutic agents, thrush manifests itself as a serious infection capable of dissemination (Figs. 27.59 and 27.60).

Candida glabrata (synonym *Nakaseomyces glabrata*) is also a common species that causes disease and accounts for many urinary yeast isolates. It may now be second only to *C. albicans* in superficial and invasive yeast infections in North America and Europe. Infections associated with *C. glabrata* tend to be aggressive in patients with multiple comorbidities and may be difficult to treat with traditional antifungal therapy (e.g., amphotericin B and fluconazole). This organism has different sugar assimilation patterns, notably rapid assimilation of trehalose, from those of *C. albicans* and therefore can be easily differentiated.

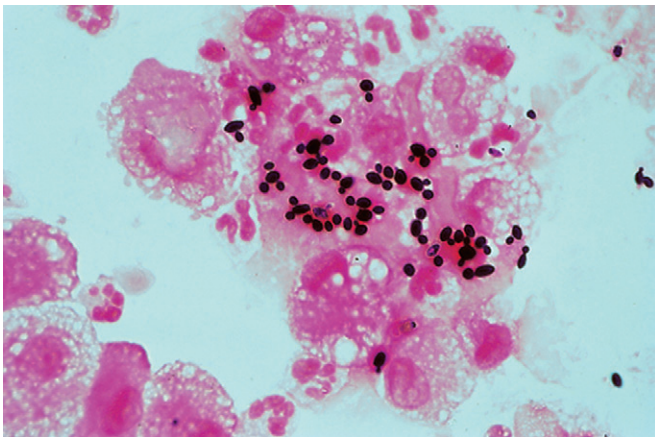


Fig. 27.59 Bronchoalveolar lavage, cytocentrifuge preparation, Gram stain, light microscopy. Gram-positive yeast with buds. Morphotype consistent with *Candida* spp. (×1000).

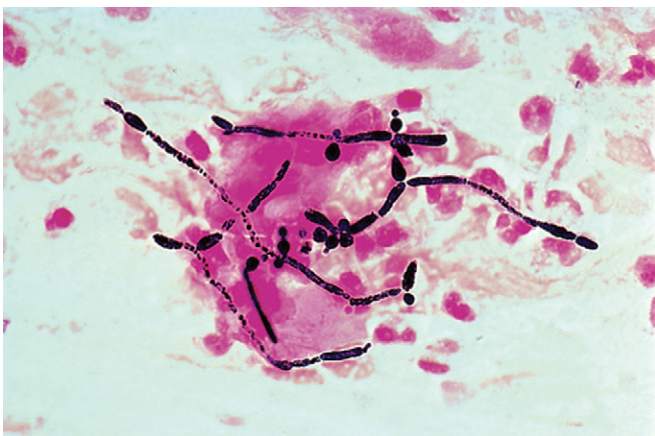


Fig. 27.60 Expectorated sputum smear, Gram stain, light microscopy. Gram-positive pseudohyphae. Morphotype consistent with *Candida* spp. (×1000).

Recently, *C. auris* has emerged as a multidrug-resistant yeast linked to high mortality rates associated with healthcare-associated infections worldwide. The yeast is likely transmitted from patient to patient in health care settings and has caused several outbreaks in numerous countries. The Centers for Disease Control and Prevention (CDC) recommends that a contact prevention protocol be followed for residents in nursing homes colonized or infected with *C. auris* and that they be housed in single rooms, if available. *C. auris* has been misidentified as several other yeast species. MALDI-TOF mass spectrometry assays can identify this species.

Other notable species of *Candida* include *C. krusei* (teleomorph *Pichia kudriavzevii*) and *C. tropicalis*. In addition, *C. parapsilosis* has become a major cause of outbreaks of healthcare-associated infections. These isolates are identified by the differences in their carbohydrate assimilation patterns and other secondary testing procedures, such as MALDI-TOF mass spectrometry. Table 27.7 shows important differentiating characteristics among *Candida* spp. and other yeasts.

Cryptococcus

Cryptococcus spp. are important causes of meningitis, pulmonary disease, and septicemia. Members of the *C. neoformans* species complex (composed of *C. neoformans* and *C. deneoformans*), the most notable pathogens in this genus, is a major cause of opportunistic infection in patients with AIDS. These organisms are commonly found in soil contaminated with pigeon droppings and is most likely acquired by inhalation. Members of the *C. gattii* species complex (*C. gattii*, *C. bacillisporus*, *C. deuterogattii*, *C. tetragattii*, and *C. decagattii*) are emerging pathogens, particularly in the Pacific Northwest of the United States and Canada. Infections caused by this species are similar to those caused by *C. neoformans* species complex, targeting primarily immunocompromised patients. However, *C. gattii* can also cause disease in immunocompetent hosts, and it is important to distinguish the two species because the clinical course and treatment outcomes can be different.

Cryptococcus spp. produce a capsule (Fig. 27.61) that produces the characteristic mucoid colony. The capsule can be detected surrounding the budding yeast in CSF with the aid of India ink or nigrosin (Fig. 27.62). The background fluid is black, and clear unstained halos are seen around individual yeast cells. Because of its low sensitivity, the use of India ink preparation is being replaced by cryptococcal antigen tests.

The cryptococcal antigen assays and an easy-to-use lateral-flow assay are recommended for routine use in clinical microbiology laboratories. The antigen assays detect both *C. neoformans* and *C. gattii* species complexes in CSF and serum.

Cryptococcus spp. are noted for producing blastoconidia only, without producing true hyphae or pseudohyphae on cornmeal agar. All species of the genus are urease positive, and the nitrate reaction differs. Sugar assimilations differ among the species (see Table 27.7). It is extremely difficult to distinguish members of the *C. neoformans* and *C. gattii* species complexes from each other. A key laboratory characteristic is that members of the *C. gattii* species complex use glycine as a sole carbon and nitrogen source in the presence of canavanine, whereas *C. neoformans* does not. Canavanine glycine bromothymol blue agar is commercially available for this purpose and can be used to help differentiate between these species complexes.

Table 27.7 Differentiating characteristics of yeasts

	Growth at			Cornmeal agar					
	37°C	42°C	45°C	Pseudohyphae	True hyphae	Arthroconidia	Cyclohexamide	Urea	Nitrate
Candida									
<i>C. albicans</i>	+	+	+	+	+	–	R	–	–
<i>C. auris</i>	+	+	–	–	–	–	S	–	–
<i>C. dubliniensis</i>	+	–	–	+	+	–	R	–	–
<i>C. glabrata</i> (syn. <i>Nakaseomyces glabrata</i>)	+	+	+	–	–	–	S	–	–
<i>C. guilliermondii</i> (teleomorph name <i>Meyerozyma guilliermondii</i>)	+	+	–	+	–	–	R	–	–
<i>C. krusei</i> (teleomorph name <i>Pichia kudriavzeii</i>)	+	+	–	+	–	–	S	V	–
<i>C. lusitanae</i> (teleomorph name <i>Clavispora lusitanae</i>)	+	+	+	+	–	–	V	–	–
<i>C. parapsilosis</i>	+	–	–	+	–	–	S	–	–
<i>C. stellatoidea</i> (<i>Candida albicans</i> var. <i>stellatoidea</i>)	+	+	+	+	+	–	S	–	–
<i>C. tropicalis</i>	+	+	+	+	–	–	V	–	–
Cryptococcus									
<i>C. albidus</i> (<i>Naganishia albidus</i>)	–	–	–	–	–	–	S	+	+
<i>C. neoformans</i>	+	–	–	–	–	–	S	+	–
<i>C. gattii</i>	+	–	–	–	–	–	R	+	–
<i>Trichosporon</i> spp.	+	V	–	+	+	+	R	+	–

+, Positive; –, negative; R, resistant; S, sensitive; syn., synonym; V, variable; current names in parentheses; var., variant.

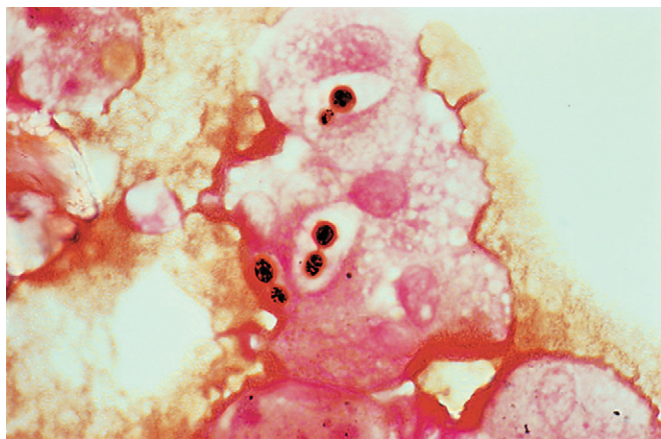


Fig. 27.61 Bronchoalveolar lavage, cytocentrifuge preparation, Gram stain, light microscopy. Red blood cells present. Gram-variable yeast with capsules. Morphology suggests *Cryptococcus* spp. (×1000) (see Fig. 27.68).

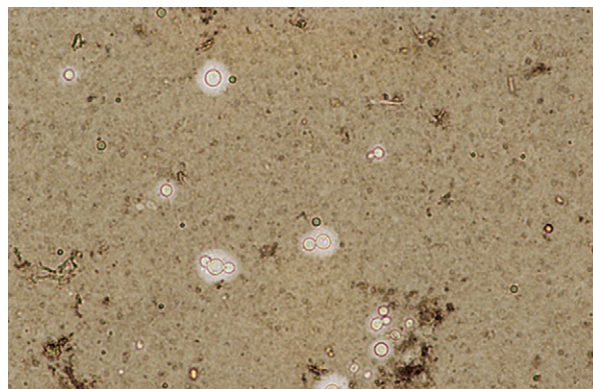


Fig. 27.62 India ink preparation is used primarily to examine cerebrospinal fluid for the presence of the encapsulated yeast *Cryptococcus neoformans*. This is an India ink preparation from an exudate containing encapsulated budding yeasts (×400).

Rhodotorula

Rhodotorula spp. are noted for their bright salmon pink color. They resemble the cryptococci because they bear a capsule and are urease positive. Some species are also nitrate positive. They are not common agents of disease but have been known to cause opportunistic infections.

Pneumocystis

Pneumocystis can inhabit the lungs of many mammals. *Pneumocystis carinii* was originally classified with the protozoa, but nucleic acid sequencing showed conclusively that the organism is a fungus. It is apparent from nucleic acid studies that *P. carinii* is not a single species. *P. carinii* is the

species most commonly found in rats, and *P. jirovecii* is the species most often recovered from humans.

Clinical manifestations

Pneumocystis infection is acquired early in life; serologic studies have shown that most humans have antibodies or antigens by 2 to 4 years of age. In immunocompetent individuals, infection is asymptomatic; however, in immunocompromised patients, serious life-threatening pneumonia can develop. *Pneumocystis* initially was identified as the causative agent in interstitial plasma cell pneumonia seen in malnourished or premature infants. Since the early 1980s, it has remained one of the primary opportunistic infections found in patients with AIDS. A high incidence of disease also results from the use of immunosuppressive drugs in patients with malignancies and in organ transplant recipients. Underlying defects in cellular immunity apparently make patients susceptible to clinical infection with the organism.

Patients infected with *Pneumocystis* may have nonproductive cough, difficulty breathing, and a low-grade fever. Chest x-rays can be normal or show a diffuse interstitial infiltrate. The immune response to the organism after it attaches to and destroys alveolar cells is partly responsible for this radiographic pattern. When the infiltrate is examined, it is found to contain cells from the alveoli and plasma cells.

Life cycle

Pneumocystis is a nonfilamentous fungus. Terminology referring to the various life cycle forms, however, is reflected in the fact that it was first considered a protozoan. The life cycle of *Pneumocystis* has three stages: the trophozoite, which is 1 to 5 μm in size and is irregularly shaped; the precyst, which is 5 to 8 μm in size; and the cyst, which is a thick-walled sphere of about 8 μm containing up to eight intracystic bodies.

Transmission of the organism is known to occur through the respiratory route, with the cyst being the infective stage. The spores or intracystic bodies are released from the cyst in the lung, and these trophic forms multiply asexually by binary fission on the surface of the epithelial cells (pneumocyte) lining the lung. Sexual reproduction by trophozoites also occurs, first producing a precyst and then the cyst containing spores or intracystic bodies.

Laboratory diagnosis

Traditionally, diagnosis was made by finding the cyst or trophozoite in tissue obtained through open lung biopsy. Specimens now used include bronchoalveolar lavage (BAL) fluid, transbronchial biopsy specimens, tracheal aspirate, pleural fluid, and induced sputum. Sputum, however, is the least productive specimen. Lavage and sputum specimens are often prepared using a cytocentrifuge (Fig. 27.63).

Histologic stains, such as Giemsa and GMS stains, are used. With the methenamine silver stain, the cyst wall stains black. Cysts often have a punched-out ping-pong ball appearance. Fig. 27.64A shows the characteristic black-staining cyst of *Pneumocystis jirovecii* with methenamine silver stain. With the Giemsa stain, the organism appears round, and the cyst wall is barely visible. Intracystic bodies are seen around the interior of the organism. Fig. 27.64B shows the cyst stained with Giemsa stain. The cyst wall does not pick up the stain, but the nuclei of all forms stain pink, and the intracystic bodies can be demonstrated as a circular arrangement within the cyst.

Calcofluor white can be used to screen specimens for *Pneumocystis* and other fungi. This stain detects any

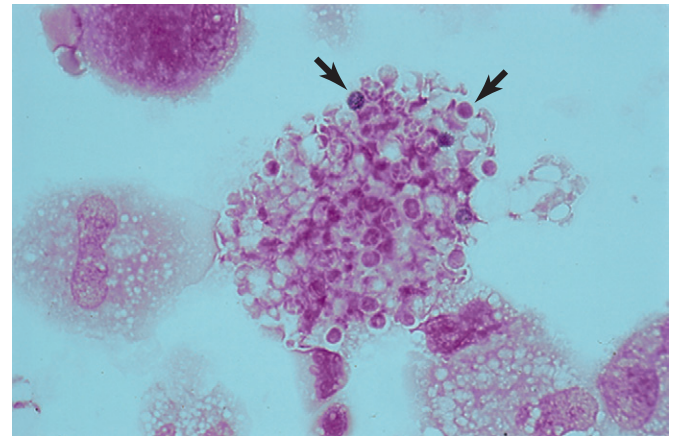


Fig. 27.63 Bronchoalveolar lavage, cytocentrifuge preparation, Gram stain, light microscopy, high power view. Purulence, none. Local materials, moderate. Alveolar cast composed of Gram-negative matrix and intracystic bodies (arrows) ($\times 1000$). Morphology consistent with *Pneumocystis jirovecii* (see Fig. 27.65).

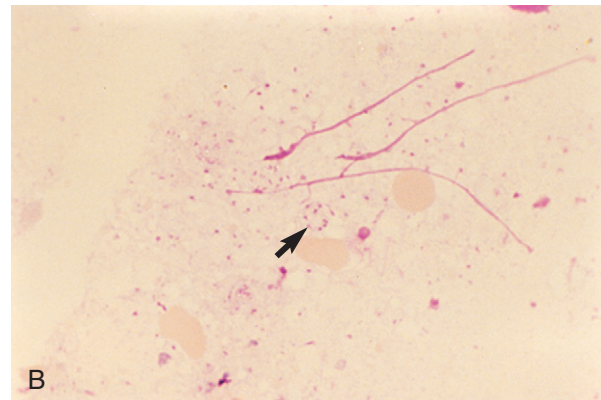
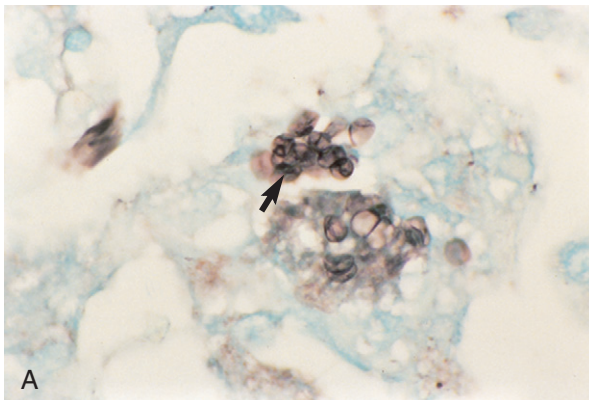


Fig. 27.64 A, *Pneumocystis jirovecii* cysts (silver stain). B, *P. jirovecii* (Giemsa stain). Note the circular arrangement of intracystic bodies within a faint outline of the cyst wall in the center of the field (A, B, $\times 1000$).

organism that contains chitin in its cell wall. Fungi, yeasts, and *Pneumocystis* will fluoresce with a blue-white color when stained and viewed microscopically with ultraviolet light (Fig. 27.65). Immunofluorescent monoclonal antibody stains are commercially available and are widely used.

Laboratory diagnosis of fungi

Safety issues

Because of the additional hazard of airborne conidia, a class 2 biological safety cabinet should be used to reduce exposure of personnel to fungal elements. Specimen processing and plating must be performed in a properly maintained and operating biological safety cabinet. The use of an enclosed electric incinerator is recommended to eliminate the hazards of open gas flames and to contain aerosolized particles emitted when loops or needles are incinerated. The cabinet should be checked daily to see that none of the air flow inlets or outlets is blocked by supplies, incinerators, or waste-disposal containers.

Use of Petri dishes in the mycology laboratory is hazardous; screw-top tubes (slants) are recommended instead for filamentous fungi. The chance for the release of airborne conidia is less likely with tubed media. Screw-capped tubes also tend to reduce dehydration of media and are more easily handled and stored compared with Petri dishes. However, Petri dishes have a larger surface area for colony isolation and are easier to manipulate when making preparations for microscopic examination. The edges of Petri dishes can be sealed with Parafilm or tape to reduce the risk of releasing airborne conidia.

Specimen collection, handling, and transport

Collection of appropriate specimens is the primary criterion for accurate diagnosis of mycotic infections. All specimens for mycology should be transported and processed as soon as possible. Because many pathogenic fungi grow

slowly, any delay in processing compromises specimen quality and decreases the probability of isolating the causative agent as a result of overgrowth by contaminants. In addition, all laboratories should maintain a protocol for the rejection of unsatisfactory or improperly labeled specimens.

Although almost any tissue or body fluid can be submitted for fungal culture, the most common specimens are respiratory secretions, hair, skin, nails, tissue, blood, bone marrow, and CSF. Table 27.8 presents the predominant culture sites for recovery of the causative agents of fungal diseases. Fig. 27.66 presents a schematic guideline to assist in making a diagnosis of a mycosis.

Hair, skin, or nails submitted for dermatophyte culture are generally contaminated with bacteria, rapidly growing fungi, or both. With these types of specimens, primary isolation medium should contain antimicrobial agents. The following procedures are recommended for collecting and processing clinical samples submitted for fungal studies.

Hair

The Wood lamp emits ultraviolet light of wavelength greater than 365nm and can be useful in identifying infected hairs. Hairs infected with fungi such as *M. audouinii* fluoresce when light from the Wood lamp is focused on the scalp. Sterile forceps should be used to pull affected hair. A less useful method involves cutting the hairs close to the scalp with sterile scissors. Hairs are placed directly into a sterile Petri dish. A few pieces of hair are inoculated onto fungal medium and incubated at 22° to 30°C.

Skin

Skin must be cleaned with 70% isopropyl alcohol before sampling. Skin samples are scraped from the outer edge of a surface lesion. A KOH wet mount is prepared with some of the scrapings; the KOH breaks down tissue, making it easier to view fungal hyphae. The remaining material is inoculated directly onto the agar.

Nails

Nails are cleaned with 70% isopropyl alcohol before the surface is scraped. Nail specimens may be submitted as scrapings or cuttings and occasionally as a complete nail. Deeper scrapings are necessary to prepare a KOH preparation and inoculate media. Sterile scissors are used to cut complete nails into small thin strips, which are used to inoculate media.

Blood and bone marrow

Blood from septicemic patients can harbor known pathogenic and opportunistic fungi, the most common being *Candida* spp. The lysis centrifugation system, the Isolator tube (Wampole Laboratories, Cranbury, NJ), is a method for the recovery of molds and dimorphic fungi. However, this method has a high contamination rate. The lysis of white blood cells (WBCs) and red blood cells releases microorganisms, which are then concentrated into sediment during centrifugation. The sediment is inoculated onto solid culture media. A biphasic system (broth and agar),

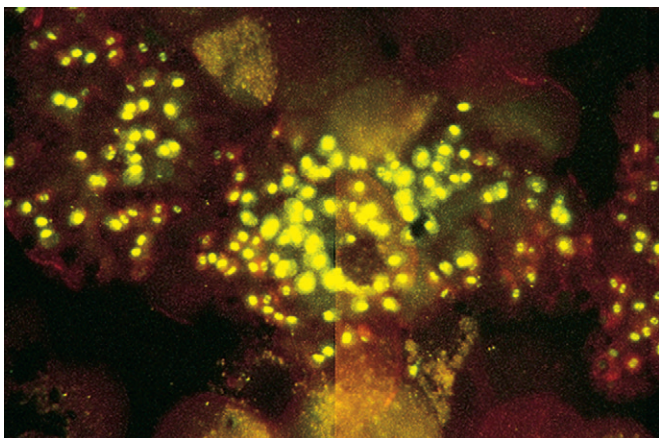
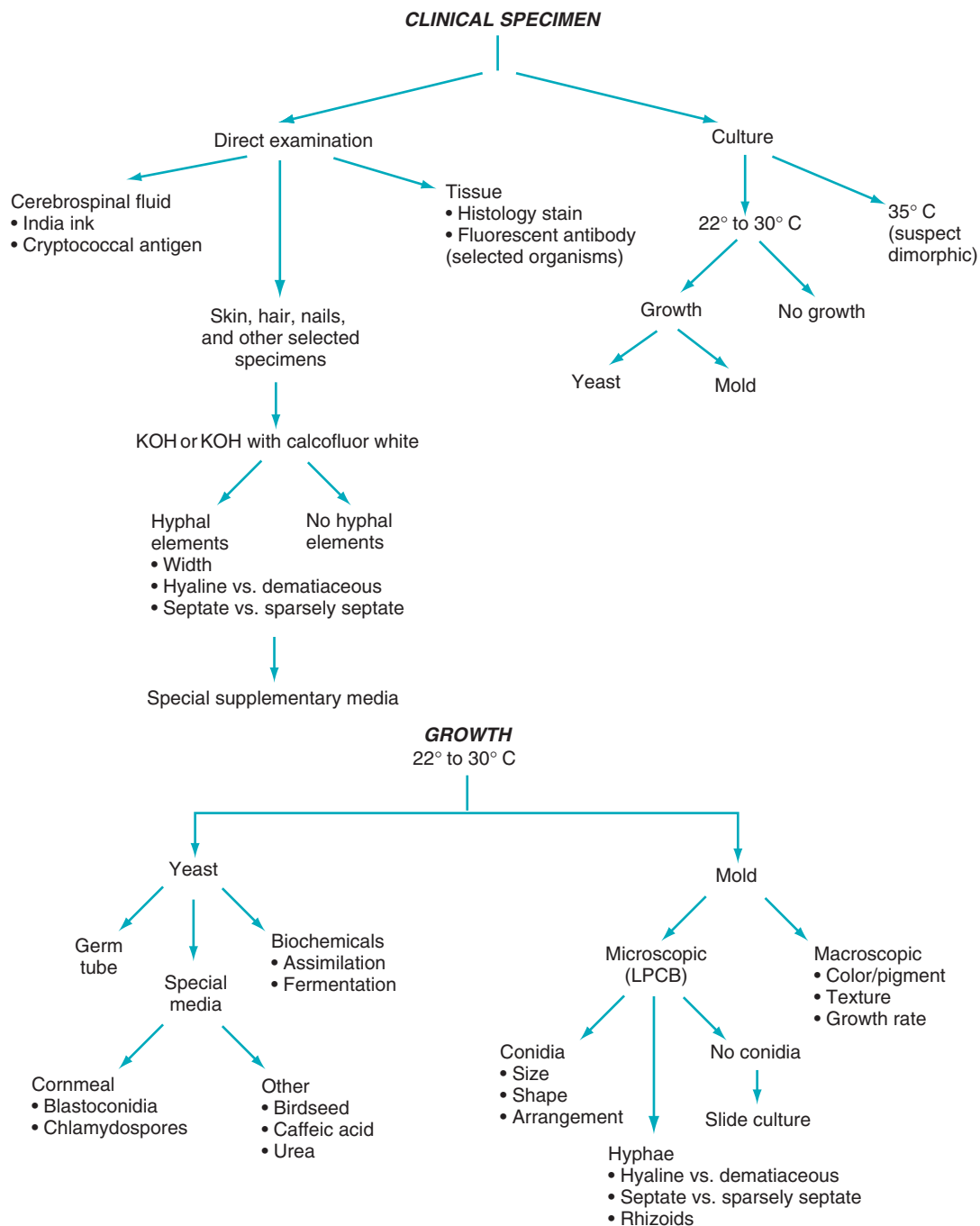


Fig. 27.65 Bronchoalveolar lavage, cytocentrifuge preparation, calcofluor white stain, fluorescence microscopy. Fluorescent cysts with coiled bodies. Morphology consistent with *P. jirovecii* ($\times 1000$).

Table 27.8 Predominant culture sites for recovery of causative agents^a

Infection	Respiratory	Blood	Bone marrow	Tissue	Skin	Mucus	Bone
Blastomycosis	+				+	+	+
Histoplasmosis	+	+	+				
Coccidioidomycosis	+				+	+	
Paracoccidioidomycosis	+				+	+	
Sporotrichosis	+			+	+	+	
Chromoblastomycosis				+	+		
Eumycotic mycetoma				+	+		
Phaeohyphomycosis				+	+		

^aOrganisms may be recovered from multiple sites in disseminated infections.Fig. 27.66 Guideline for the identification of fungal isolates. *KOH*, Potassium hydroxide; *LPCB*, lactophenol cotton blue.

such as the Septi-Chek (BD Diagnostic Systems, Sparks, MD), can also be used. Automated and continuous monitoring blood culture systems have media designed for the recovery of fungi. As with bacteriology, blood volume and blood-to-broth ratio are important for optimal recovery of fungi. Studies indicate 20 to 30 mL of blood from adults divided among two bottles and a dilution of 5- to 10-fold is ideal. Heparinized bone marrow specimens should be plated directly onto media at the bedside; use of blood culture bottles is not recommended.

Cerebrospinal fluid

CSF and other sterile body fluids should be concentrated by centrifugation before inoculation. Part of the concentrate is used for India ink preparation or other assays for *Cryptococcus* (e.g., latex agglutination assay, EIA or lateral flow assay, as described later), and the remainder is inoculated onto media. If more than 5 mL is submitted, the CSF may be filtered through a membrane filter and portions of the filter placed on media. Use of media with antimicrobial agents should not be needed because CSF is normally sterile.

Abscess fluid, wound exudates, and tissue

Using a dissecting microscope, abscess fluid and exudate from wounds can be examined for the presence of granules. If no granules are present, the material may be plated directly onto the media. Tissue should be gently minced before inoculation. Grinding of tissue has been recommended, but this process might destroy fragile fungal elements, particularly if a mucormycete is present. Although grinding of tissue might be necessary for KOH and calcofluor white preparations, it should not be done unless sufficient tissue is present for mincing and grinding. When large sections of tissue are submitted, suspicious areas, such as purulent or discolored sections, are selected for mincing and grinding before subsequent culture.

Respiratory specimens

Because many fungal infections have a primary focus in the lungs, lower respiratory tract secretions (e.g., sputum, trans-tracheal aspirates) and lavage fluids (e.g., BAL) are commonly submitted. Patients should obtain sputa from a deep cough shortly after arising in the morning. If the patient cannot produce sputum, a nebulizer may be used to induce sputum. All sputum specimens should be collected in a sterile, screw-top container.

If the material is not too viscous, the specimens can be inoculated onto media with a sterile pipette. With viscous materials, such as a thick tracheal aspirate, a synthetic swab (e.g., Dacron) may be used to inoculate the material onto the media, or preferably the specimen can be digested with the mucolytic agent *N*-acetyl-L-cysteine and concentrated before inoculation. In addition to nonselective media, a medium with antimicrobial agents should be used to prevent bacterial overgrowth. A KOH preparation should also be made. Oropharyngeal candidiasis is easily diagnosed with direct smear and culture. Nasal sinus specimens obtained surgically can be plated directly to media containing antimicrobial agents except for cycloheximide, which can inhibit some fungi.

Urogenital and fecal specimens

Laboratory scientists often receive urine, feces, and vaginal secretions as specimens for bacteriologic culture; on occasion, these specimens grow a yeast that requires identification. Urine submitted specifically for fungal culture should be centrifuged and the sediment used to make smears for microscopic examination and to inoculate media. Quantification of yeast, as performed for bacteria, is not useful, and 24-hour specimens are unacceptable. A first-morning voided urine specimen is preferred.

Direct microscopic examination of specimens

Direct examination of clinical material for fungal elements serves several purposes. First, it helps provide a rapid report to the primary care provider, which may result in the early initiation of treatment. Second, in some cases, specific morphologic characteristics provide a clue to the genus of the organism. In turn, any special media indicated for species identification can be inoculated immediately. Third, direct examination might provide evidence of infection despite negative culture results. Such a situation can occur with specimens from patients who are receiving antifungal therapy, which may inhibit growth in vitro even though the infection can still be present in the patient.

Although Gram-stained smears of clinical samples examined in the routine microbiology laboratory often give the first evidence of infections with bacteria and yeasts, other direct stains give more specific information about mold infections. The types of direct examination used in identification of fungal infections include wet preparations, such as KOH, India ink, and calcofluor white stain. Histologic stains for tissue may also be useful.

KOH preparation

A 10% to 20% solution of KOH is useful for detecting fungal elements embedded within skin, hair, nails, and tissue (Fig. 27.67). In this procedure, a drop of the KOH preparation is added to a slide. Nail scrapings, hair, skin scales, or thin slices of tissue are added to the drop, and a coverslip is placed

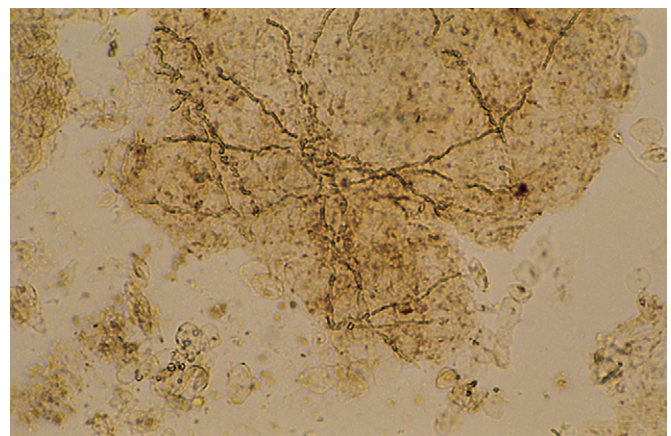


Fig. 27.67 Skin scales, scrapings, potassium hydroxide wet preparation, light microscopy ($\times 400$). Hyphae present, septate, thin. Morphology suggests dermatophyte.

on top. The slide is then gently heated and then allowed to cool for approximately 15 minutes. The KOH and heat break down the keratin and skin layers, revealing more clearly fungi present in the specimen. Interpreting KOH slides remains difficult, and fungal elements can still evade detection.

Modifications of the KOH test can provide easier and more reliable results. These preparations incorporate dimethyl sulfoxide (DMSO) and a stain into the KOH solution. The DMSO facilitates more rapid breakdown of cellular debris without requiring heat, while the stain is taken up by fungal elements, making them readily visible on microscopic examination of the slide preparation.

Potassium hydroxide with calcofluor white

A drop of calcofluor white can be added to the KOH preparation before adding a coverslip. Calcofluor white binds to polysaccharides present in the chitin of the fungus or to cellulose. Fungal elements fluoresce apple green or blue-white, depending on the combination of filters used on the microscope; therefore any element with a polysaccharide skeleton fluoresces (Figs. 27.68 to 27.70). Because calcofluor white stains other structures, the actual fungal structure must be seen before a positive preparation is reported (Fig. 27.71). Care must also be taken when using this process because much variability exists among manufacturers and even in different lots of the stain prepared by the same manufacturer.

India ink

India ink or nigrosin preparations can be used to examine CSF for the presence of the encapsulated yeast *Cryptococcus*. A drop of India ink is mixed with a drop of sediment from a centrifuged CSF specimen, and the preparation is examined on high magnification ($\times 400$). With this negative stain, budding yeast cells surrounded by a large clear area against a black background are presumptive evidence of *Cryptococcus* (see Fig. 27.62). WBCs and other artifacts can resemble encapsulated organisms; therefore careful examination is necessary. Many laboratories, however, now use a cryptococcal antigen assay (see the “Cryptococcal Antigen” section later in this chapter) in place of the India ink examination.

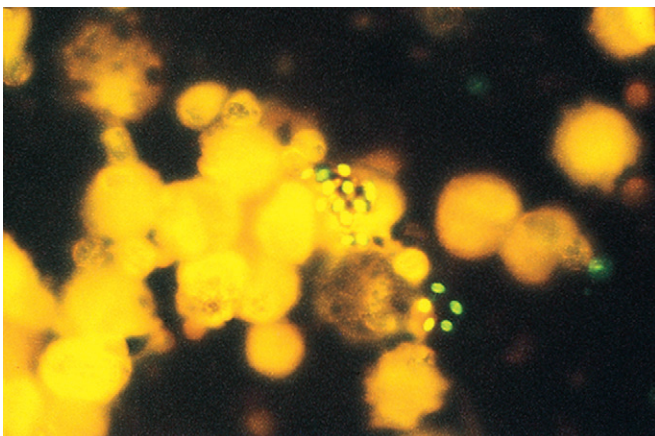


Fig. 27.68 Bronchoalveolar lavage, cytocentrifuge preparation, calcofluor white stain, fluorescence microscopy ($\times 1000$). Fluorescent yeast, small, with capsules. Morphology suggests *Cryptococcus neoformans*. Impression: cryptococcosis.

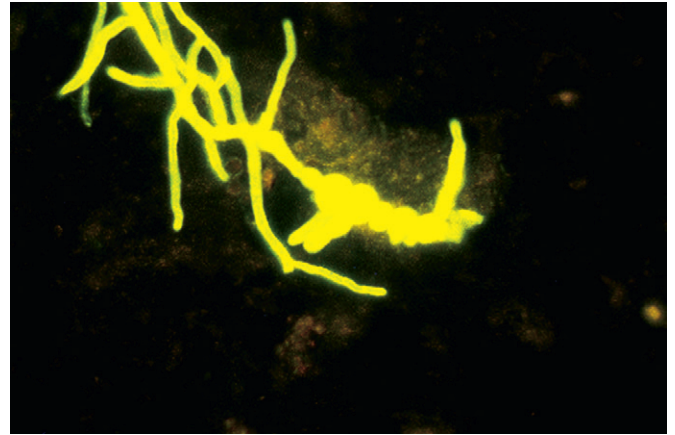


Fig. 27.69 Soft tissue abscess, smear, calcofluor white stain, fluorescence microscopy ($\times 1000$). Fungal hyphae, septate, branched chlamydospores. Impression: mycosis. These hyphae were not clearly visible on the Gram stain smear, but they stain brightly here. A dermatophyte was isolated in culture.

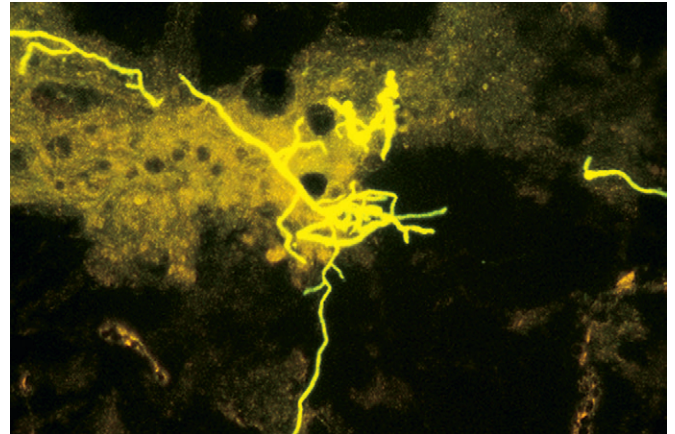


Fig. 27.70 Skin scales from scrapings, calcofluor white stain, fluorescence microscopy ($\times 1000$). Hyphae, thin. Morphology suggests dermatophyte. Impression: dermatophytosis.

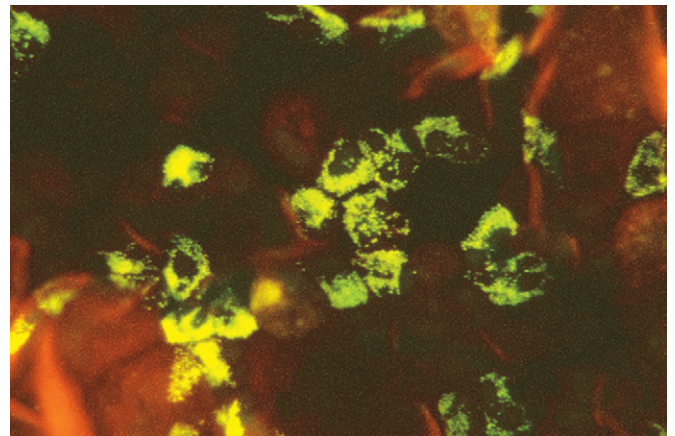


Fig. 27.71 Expectorated sputum, smear, calcofluor white stain, fluorescence microscopy ($\times 400$). Eosinophils. These cells are other structures that stain with calcofluor white. The granules from ruptured eosinophils stain brightly and should not be interpreted as remnants of fungi or parasites.

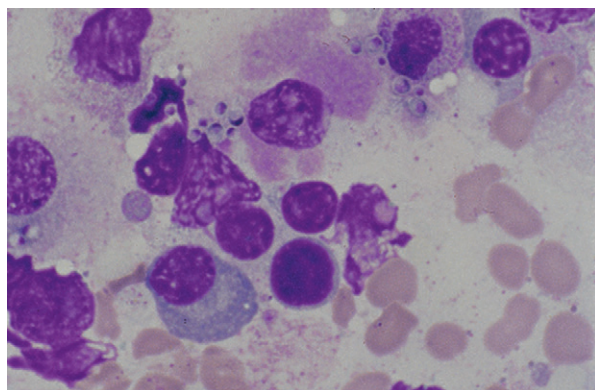


Fig. 27.72 Bone marrow stained with Giemsa stain showing the yeast phase of *Histoplasma capsulatum* (×1000).

The performance of these assays has improved over the years, and they are now recommended over the India ink preparations in screening CSF for *Cryptococcus* spp.

Tissue stains

Common tissue stains used in the histology department for detection of fungal elements include the periodic acid–Schiff (PAS), GMS, hematoxylin and eosin (H&E), Giemsa, and Fontana-Masson stains. Giemsa stain is used primarily to detect *Histoplasma* in blood or bone marrow (Fig. 27.72). PAS stain attaches to polysaccharides in the fungal wall and stains fungi pink. The Fontana-Masson method stains melanin in the cell wall and identifies the presence of phaeoid fungi. Table 27.9 lists the characteristic fungal reactions seen with selected stains.

Case check 27.2

When hyphal elements are seen in tissue sections, the disease is frequently attributed to *Aspergillus* spp. Although *Aspergillus* is the most common mold causing disease in the immunocompromised patient, dozens of other fungi, such as *Fusarium* in the Case in Point, give the same appearance. To document infection, the mold must be seen in tissue and grown in culture. When hyphae are not seen in tissue, the positive culture may be caused by a contaminant, and conversely, when hyphae are seen in tissue but not grown in culture, the causative agent cannot be determined.

Isolation methods

Culture media

In general, fungi do not share the broad range of nutritional and environmental needs that characterize bacteria, so relatively few types of standard media are needed for primary isolation. These include Sabouraud dextrose agar, Sabouraud dextrose agar with antimicrobial agents, potato dextrose agar or potato flakes agar, and BHI agar enriched with blood and antimicrobial agents. Gentamicin or chloramphenicol and cycloheximide are the antimicrobials usually included with fungal media. Gentamicin and chloramphenicol inhibit bacterial growth, whereas cycloheximide inhibits bacteria and many of the environmental fungi typically considered contaminants.

Table 27.9 Staining characteristics of fungi

Stain	Color of fungal element	Background color
Periodic acid–Schiff	Magenta	Pink or green
Grocott–Gomori methenamine silver	Black	Green
Giemsa	Purple-to-blue yeast with clear halo (capsule)	Pink to purple
India ink	Yeast with clear halo (capsule)	Black
KOH	Refractile	Clear
KOH–calcofluor white	Fluorescent	Dark
Fontana-Masson	Brown	Pink to purple

KOH, Potassium hydroxide.

The pH of the Emmons modification of Sabouraud dextrose agar is close to neutral and is a more efficient medium for primary isolation compared with the original formulation. Table 27.10 shows the expected growth results with some of the standard fungal media. Selective chromogenic agars, such as CHROMagar *Candida* (CHROMagar, Paris, France) and CHROMID *Candida* (bioMérieux, Durham, NC), provide a rapid preliminary identification.

Fungal media can be poured into Petri dishes or large test tubes to make slants. Petri dishes have the advantage of a larger surface area but are more prone to dehydration because of the prolonged incubation necessary for the recovery of some fungi. Petri dishes must be poured thicker than a standard medium for bacterial growth. The plates can be sealed with tape or Parafilm or sealed in semi-permeable bags to minimize dehydration and prevent the spread of fungal spores. Several examples of semi-permeable shrink seal bands are commercially available as well. Tubed media have the advantage of being safer to handle and less susceptible to drying. Fungal plates and tubes should be opened only in a biological safety cabinet.

Incubation

Most laboratories routinely incubate fungal cultures at room temperature or at 30°C. Fungi grow optimally at these temperatures, whereas bacteria have a slower growth rate. If the causative agent suspected is a dimorphic fungus, cultures should also be incubated at 35°C. Cultures are generally maintained for 4 to 6 weeks and should be examined twice weekly for growth. Mucorales, such as *Mucor* and *Rhizopus* spp., grow rapidly and may fill the tube or Petri dish with aerial mycelium within a few days, whereas more slowly growing organisms, such as *Fonsecaea* or *Phialophora* spp., might require 2 weeks or longer before growth is seen.

Information that should be recorded about an isolate includes the number of days until the first visible growth and the number of days required to see fruiting structures, whether mold or yeast forms are recovered, the media on which the fungus is isolated, the temperature at which growth occurs, and the morphology of the colonies.

Table 27.10 Summary of primary fungal culture media

Medium	Expected growth results
Incubated at 22°C	
SDA	Initial isolation of pathogens and saprobes Dimorphic fungi may exhibit their mycelial phase
SDA with antimicrobials ^a	Saprobes generally inhibited on this medium Dermatophytes and most of the fungi considered primary pathogens grow
BHI agar	Initial isolation of pathogens and saprobes
BHI agar with antimicrobials	Recovery of pathogenic fungi Dermatophytes not usually recovered
Inhibitory mold agar ^b	Initial isolation of pathogens except dermatophytes
SDA with chloramphenicol and cycloheximide ^c	Primary recovery of dermatophytes
At 37°C	
SDA	The yeast form of dimorphic fungi, and other organisms grow Dermatophytes grow poorly
BHI agar with blood	Yeasts, such as <i>Cryptococcus</i> , grow well The yeast form of <i>Histoplasma</i> takes up some of the heme pigment in the medium and becomes light tan, with a grainy, wrinkled texture

BHI, Brain-heart infusion; *SDA*, Sabouraud dextrose agar (4% glucose) or Emmons' modification with 2% glucose.
^aThe antimicrobial agents generally used are cycloheximide and chloramphenicol.
^bUsually contains chloramphenicol and is used for cultivation of cyclohexamide sensitive fungi (e.g., *Cryptococcus* spp. and *Candida* spp.). Yeast extract and glucose provide nutrients.
^cSeveral commercial formulations are available (e.g., Mycobiotic agar [Remel, Lenexa, KS] and Mycosel [BD Diagnostic Systems, Sparks, MD]).

Fungi identification

Although the number of fungal species described exceeds 100,000, the number known to cause human disease is a small fraction of this number, and although the number of species that are routinely seen causing infection is quite low, new species are continually being implicated. Most diseases are caused by a handful of species, making identification, at least to the genus level, fairly easy. The traditional starting place is to decide whether the isolate is a yeast or a mold. However, molds do not always produce structures that will facilitate identification. Often, cultures from clinical samples are sterile (hyphae only) or atypical.

None of the following tests alone is sufficient for proper identification, but when used together, accurate identification is often accomplished easily. Molecular and proteomic tests, whether culture based (DNA hybridization using probes, DNA sequencing and MALDI-TOF mass spectrometry) or PCR based, when used with these test procedures, enable the laboratory scientist to identify the most commonly encountered fungal isolates.

Macroscopic examination of cultures

Once an organism has grown, colonies must be examined for macroscopic characteristics. Gross morphologic traits, such as

color, texture, and growth rate, are initial observations that should be made. Pigment on the reverse side of the colony or in the aerial mycelium can be noted but is not always helpful, especially with the phaeoid fungi.

Microscopic examination

The most common procedure for microscopic examination is direct mounting of the fungal isolate. This is achieved by preparing a tease mount or cellophane tape mount. Many fungi routinely recovered can be identified by either of these two methods. Because of the risk of airborne conidia, these slides must be prepared in a biological safety cabinet. When fungi are atypical or an uncommon species is recovered, a slide culture should be prepared. Tease and tape mounts typically disturb conidia, preventing viewing of how they are formed, whereas slide cultures provide a more intact specimen. Fruiting structures, as well as conidial arrangement, are better observed by this method. The following microscopic characteristics should be observed:

- Septate versus sparsely septate or aseptate hyphae
- Hyaline or phaeoid hyphae
- Reproductive (fruiting) structures
- The types, size, shape, and arrangement of conidia/ascospores

Tease mount

For the tease mount, teasing needles or sterile applicator sticks are used to remove a portion of the mycelium from the middle third of the colony. The mycelia are placed in a drop of lactophenol cotton blue (LPCB) on a slide and gently teased apart. A modification of this procedure is more practical and permits retention of more of the fruiting structure. The mycelia are placed into a drop of LPCB on a slide but not teased apart, a coverslip is added, and the slide is examined microscopically at low and high magnifications.

LPCB is used to fix and stain tease or tape mounts from cultures. The combination of lactic acid, phenol, and the dye kills, preserves, and stains the organism. The hyphae take up the LPCB, but the stain does not work well with the phaeoid fungi because they retain their dark color. The major disadvantage of this procedure is the disruption of conidia during the teasing process.

Cellophane tape preparation

Cellophane tape preparations involve gently touching the surface of the colony with a piece of clear tape, sticky side down, and then removing it. The tape should not be pressed into the colony but should just gently touch the surface. Frosted tape does not work because the fungi are not visible through this type of tape. The tape is placed onto a drop of LPCB on a slide and examined. An advantage of this procedure is that the conidial arrangement is retained. Major disadvantages are the potential for contamination of the colony and the temporary nature of this preparation. Tape preparations should be read within 30 minutes and then discarded. A coverslip is not needed if the cellophane tape technique is used because the tape serves as a coverslip.

Slide culture

Slide cultures are useful for demonstrating the natural morphology of fungal structures and for encouraging conidiation

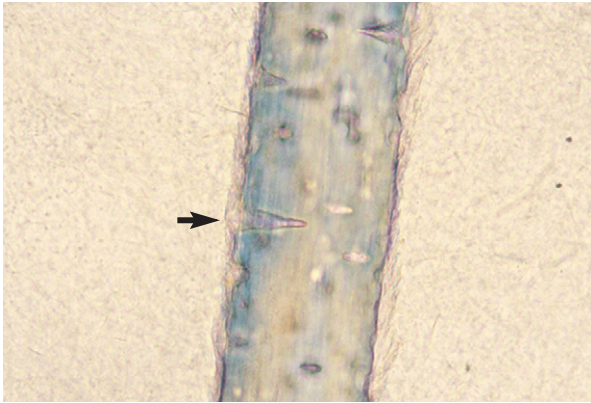


Fig. 27.73 A positive hair perforation test result shows penetration of the fungal agent in the hair shaft (arrow). This is the typical reaction by *Trichophyton mentagrophytes*, whereas *Trichophyton rubrum* causes only surface erosion of hair shaft (unstained, $\times 1000$).

in some poorly fruiting fungi. The slide culture can be preserved for a prolonged period, making those of known isolates useful for future comparison against isolates awaiting identification. Several methods have been devised for constructing slide cultures (see [Appendix D](#) on Evolve).

Miscellaneous tests for the identification of molds

Hair perforation test

In the hair perforation test, sterile 5- to 10-mm hair fragments are floated on sterile water supplemented with a few drops of sterile 10% yeast extract. Conidia or hyphae from the dermatophyte in question are inoculated onto the water surface. Hair shafts are removed and microscopically examined in LPCB at weekly intervals for up to 1 month. *T. rubrum* usually causes only surface erosion of hair shafts in this test, whereas *T. mentagrophytes* typically forms perpendicular penetration or wedge-shaped pegs in the hair shafts ([Fig. 27.73](#)). This test may be used to distinguish penetration-capable *M. canis* from *M. equinum*, which does not penetrate hair.

Urease test

Another test used to help differentiate *T. mentagrophytes* from *T. rubrum* is the 5-day urease test. Tubes of Christensen urea agar are very lightly inoculated with the dermatophyte and held for 5 days at room temperature. Most isolates of *T. mentagrophytes* demonstrate urease production, resulting in a color change of the medium from peach to bright fuchsia within that period, whereas most *T. rubrum* isolates are negative or require more than 5 days to give a positive reaction. This test is also useful for other molds and yeasts.

Thiamine requirement

Some dermatophytes cannot synthesize certain vitamins and therefore do not grow on vitamin-free media. Although several vitamin deficiencies are recognized in fungi, the test for thiamine requirement is perhaps the single most useful nutritional test for dermatophytes. Tubes of media with and without thiamine are inoculated with a tiny, medium-free portion of the colony and observed for growth after 10 to 14 days. Great care must be exercised to avoid transfer of culture medium with the inoculum because even

minuscule amounts of vitamin carried over can adequately supply the requirement and thereby result in a false growth reaction.

Trichophyton agars

Seven different *Trichophyton* agars, numbered 1 to 7, are used to determine the nutritional requirements of *Trichophyton* spp. Each of the media has different nutritional ingredients; after inoculation and incubation, growth is scored on a scale of 1 to 4. Based on the growth pattern, identification is made.

Growth on rice grains

Poorly sporulating isolates of *M. canis* can be difficult to differentiate from *M. audouinii*, a species that typically forms few conidia. Sterile, nonfortified rice is inoculated lightly with a portion of a colony. After 10 days of incubation at room temperature, the medium is observed for growth. *M. canis* and almost all other dermatophytes grow well and usually form many conidia, whereas *M. audouinii* does not grow but turns the rice grains brown.

Miscellaneous tests for the identification of yeasts

Germ tube production

The **germ tube** test is a relatively easy test to perform for the identification of yeasts. [Fig. 27.74](#) shows a schematic diagram of how the germ tube test can be used to presumptively identify yeast species. Both *C. albicans* and *C. dubliniensis* are identified with germ tube production ([Fig. 27.75](#)). The standard procedure (see [Appendix D](#) on Evolve) requires the use of serum or plasma, such as fetal bovine serum. Expired fresh-frozen plasma from the blood bank can also be used. However, this is not recommended because of the potential risk in the handling of bloodborne pathogens and because of concerns over reproducibility. Many other liquid media (e.g., BHI agar, trypticase soy broth, nutrient broth) have been used successfully as an alternative. The substrate is inoculated and then incubated at 35°C for 3 hours. Care must be taken not to incubate the test beyond 3 hours because other species are capable of forming germ tubes with extended incubation.

A presumptive identification of *C. albicans* or *C. dubliniensis* can be made when true germ tubes are present. This test provides only a presumptive identification because not all strains of *C. albicans* will be positive, and other species, in particular *C. tropicalis*, can yield false-positive results. True germ tubes lack constriction at their bases, where they attach to the mother cell. If a constriction is present at the base of a germ tube, the yeast is not either species. Such constricted germ tubes, called **pseudo-germ tubes**, are more characteristic of *C. tropicalis* ([Fig. 27.76](#)). *C. dubliniensis* is differentiated from *C. albicans* by its inability to grow at 42°C.

Carbohydrate assimilation

Sugar fermentation tests, although valuable, are time- and labor-intensive, making them impractical for use in the routine microbiology laboratory. Carbohydrate assimilation tests, however, can be readily performed as part of routine identification protocols. Assimilation tests identify which carbohydrates a yeast can use aerobically as a sole carbon source. Assimilation patterns can be determined from such methods

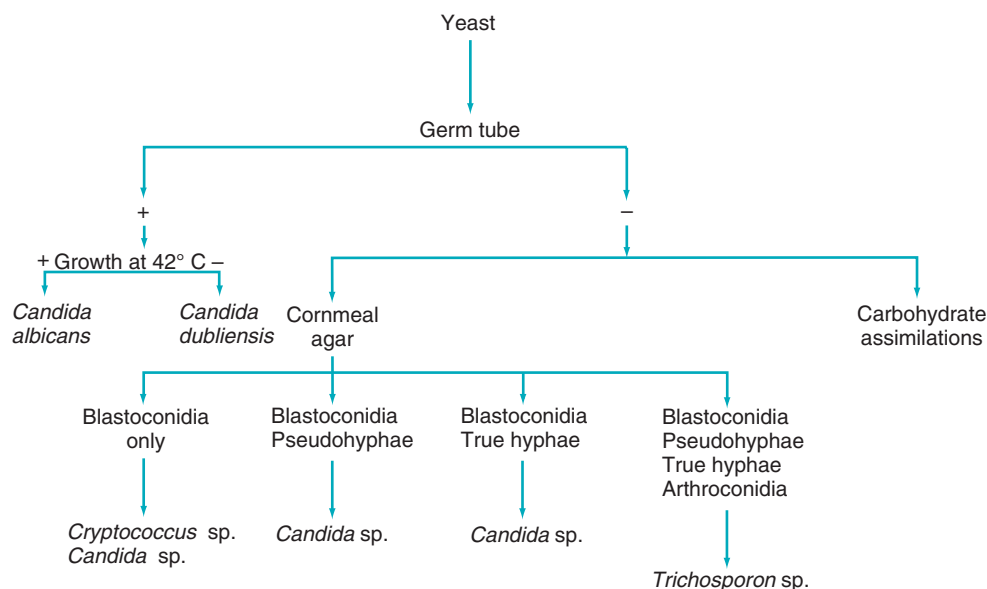


Fig. 27.74 Schematic diagram showing how the germ tube test can be used to presumptively identify yeasts.

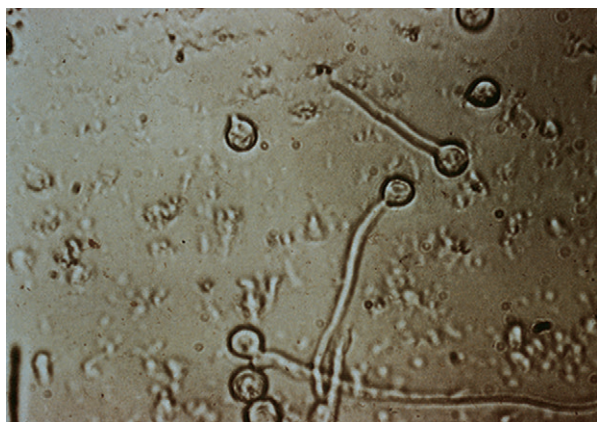


Fig. 27.75 Germ tube production by *Candida albicans*. A true germ tube has no constriction at its base (unstained, $\times 1000$).



Fig. 27.76 *Candida tropicalis* shows constriction at the base of the germ tube, called a pseudo-germ tube (Nomarski optics, $\times 1250$).

as an automated identification system or various manual procedures and commercial kits. The individual laboratory should adopt the method that can be practically implemented into its particular working environment.

Several kits are available for identification of yeasts, including the API 20C yeast identification system (bioMérieux). In this assay, a series of freeze-dried sugars are placed into wells on a plastic strip. Yeast isolates are suspended in an agar basal medium, pipetted into the wells, and incubated at 30°C for 72 hours. As sugars are assimilated, the wells become turbid with growth. Wells remain clear when the sugar is not assimilated. A code is derived from the assimilation patterns and matched against a computerized database. Identifications are accompanied by a percentage, which indicates the probability that the identification is correct. Although this test is reliable, other auxiliary testing should accompany assimilation results before a final identification is made. Automated systems are also available for yeast identification. Many of these systems use enzyme and assimilation reactions to aid in yeast identification.

Chromogenic substrates

A number of media containing chromogenic substrates are available for the presumptive identification of yeasts. CHROMagar Candida presumptively identifies *C. albicans*, *C. tropicalis*, and about 10 other species. Identification is based on different colony colors, depending on the breakdown of chromogenic substrates by the different species. About 2% to 10% of *C. albicans* isolates are not identified on these media because they form white colonies.

Cornmeal agar

Yeast morphology on cornmeal agar is useful in determining identification of yeasts (see [Appendix D](#) on Evolve). Recognition of four different types of morphology is an important clue to identification: blastoconidia, chlamydoconidia, pseudohyphae, and arthroconidia.

Blastoconidia are the characteristic budding yeast forms usually seen on direct mounts of yeasts. *C. albicans* produces chlamydoconidia (chlamydospores) along with hyphae, as shown in [Fig. 27.77](#). A chlamydoconidium is a thick-walled conidium formed within or at the end of vegetative hyphae. Pseudohyphae ([Fig. 27.78](#)) are produced when the blastoconidia germinate to form a filamentous mat.

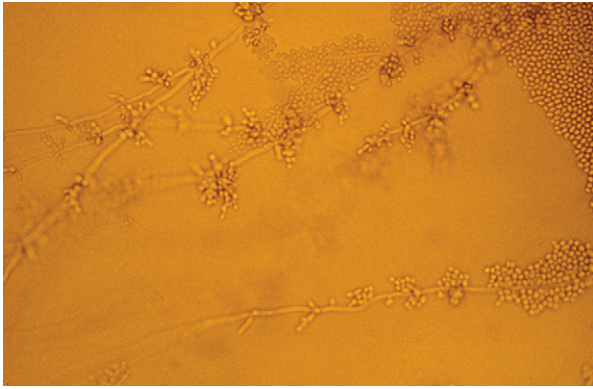


Fig. 27.77 *Candida albicans* on cornmeal agar showing typical chlamydoconidia (unstained, $\times 200$).



Fig. 27.78 Pseudohyphae occur when the blastoconidia germinate and form a filamentous mat (unstained, $\times 200$).

The cross-walls help determine whether the structures are true hyphae or pseudohyphae. Cross-walls of pseudohyphae are constricted and not true septations, whereas true hyphae remain parallel at cross-walls, with no indentation. Arthroconidia begin as true hyphae but break apart at the cross-walls with maturity. Rectangular fragments of hyphae should be accompanied by blastoconidia for an isolate to be considered a yeast.

Potassium nitrate assimilation

KNO_3 assimilation may provide useful information for separating the clinically significant yeasts. Use of the modified KNO_3 agar is a fairly rapid, easy, and accurate method to determine the ability of yeasts to use nitrate as the sole source of nitrogen. In a positive KNO_3 assimilation result, the medium turns blue, and in a negative result, the medium turns yellow. Control organisms that may be used include *Naganishia albida* (formerly *Cryptococcus albidus*) (positive) and *C. albicans* (negative).

Temperature studies

Temperature studies offer information for yeast identification. *Cryptococcus* spp. have weak growth at 35°C and no growth at 42°C . The optimal temperature for growth is about 25°C . Several *Candida* spp. have the ability to grow well at

temperatures as high as 45°C . *C. albicans* grows at 45°C , whereas *C. dubliniensis* does not. Similarly, *C. auris* is capable of growing at 40° to 42°C , whereas *C. haemulonii* and *C. duobushaemulonii*, two closely related species, are not.

Urease

Yeast isolates producing the enzyme urease can be detected easily with Christensen urea agar. The slants are inoculated and incubated at room temperature for 48 hours. Almost all clinically encountered *Candida* spp. are urease negative, whereas all *Cryptococcus* and *Rhodotorula* organisms are urease positive. Most strains of *Trichosporon* spp. are urease positive.

Diagnosis using clinical specimens and surrogate markers

(1,3)- β -D-Glucan

(1,3)- β -D-Glucan is an important component of the cell wall of various fungi, including pathogenic yeasts and molds. Major exceptions include *Cryptococcus* spp. and the Mucorales. Various assays are available, but only one, the Fungitell test (Associates of Cape Cod, Falmouth, MA), is available in the United States. This assay is a chromogenic test based on activation of the horseshoe crab coagulation cascade by (1,3)- β -D-glucan and uses amebocyte enzymes from *Limulus polyphemus*. Clinical studies have demonstrated the utility of this assay for the diagnosis of invasive fungal infections. However, various substances as well as some bacteria can lead to false-positive results. The cryptococci lack (1,3)- β -D-glucan, so they are negative. In addition, this is a panfungal assay, so a positive result does not provide information on the species that may be causing infection.

Galactomannan

The detection of galactomannan, a component of the *Aspergillus* cell wall, within plasma or BAL fluid is often used in the diagnosis of invasive infections caused by *Aspergillus* species. The detection of galactomannan is now part of the diagnostic criteria for probable invasive aspergillosis. One enzyme-linked immunosorbent assay (ELISA) method, the Platelia *Aspergillus* assay (Bio-Rad Laboratories, Hercules, CA), which uses a rat monoclonal antibody directed against an epitope in galactomannan in an ELISA format, was shown to be sensitive and specific in patients at high risk for invasive aspergillosis.

T2 magnetic resonance assay

A relatively new assay for the diagnosis of invasive candidiasis combines nuclear magnetic resonance spectroscopy with PCR to detect *Candida* cells directly in blood samples. One assay, the T2Candida assay (T2Biosystems, Lexington, MA), is approved by the U.S. Food and Drug Administration (FDA) and can detect five common *Candida* species: *C. albicans*, *C. glabrata*, *C. krusei*, *C. parapsilosis*, and *C. tropicalis*. This assay has excellent analytical sensitivity, being able to detect one to three colony-forming units of *Candida* per milliliter of blood. The results are rapidly available, usually within 4 hours in one study, compared with over 100 hours for traditional blood cultures, which is a major advantage. A research use only product is also available for *C. auris*.

Cryptococcal antigen

The detection of cryptococcal antigen, within either CSF or serum, has proven to be very useful in the diagnosis of cryptococcosis. Several available assays detect the glucuronoxylomannan component of the *Cryptococcus* antigen. These include latex agglutination assays, EIAs, and lateral flow assays. A lateral flow assay (IMMY cryptococcal lateral flow assay, Immuno-Mycologics, Norman, OK) is a rapid point-of-care dipstick test that uses a monoclonal antibody against the cryptococcal antigen. Because this assay is sensitive, is easy to perform, and does not require specialized equipment or refrigeration, the World Health Organization recommends that it be used in resource-limited settings for screening HIV-positive individuals for cryptococcosis.

Immunodiagnosis of fungal disease

Skin test reactivity to fungal antigens is sometimes used in the diagnosis of fungal allergies and infections. For example, *Aspergillus* spp. antigen extract is used for suspected allergic bronchopulmonary aspergillosis, atopic dermatitis, and allergic asthma. Skin testing for diagnosing fungal infection is valuable only if the patient has a history of a nonreactive skin test. A few antigen detection assays are commercially available for fungal pathogens including *Cryptococcus* spp. and *H. capsulatum*.

Although few are commercially available, assays to detect antibodies to fungi have also been used to help diagnose infection. Results depend on the antigen chosen and its quality. Cross-reactivity among related fungi has been reported. Double-immunodiffusion is probably the most commonly used method; however, interlaboratory discrepancies have been noted. One problem with serologic assays is the ubiquitous nature of many of the opportunistic fungi. Individuals can have detectable antibodies but not an active infection. It has also been shown that some individuals with *Aspergillus* spp. infections do not have detectable levels of antibody.

Antifungal susceptibility

Antifungal agents

Over the past few decades, fungal infections have steadily increased in incidence. This increase can be attributed to advances in medicine that have prolonged life expectancy, especially for those with chronic diseases. Advances in the area of transplantation medicine and chemotherapy, combined with immunosuppression from HIV infection, have created more patient populations susceptible to fungal infections.

Primary care providers have far fewer options for treating fungal infections compared with bacterial infections. Current options for treating fungal infection include drugs primarily from four classes: polyene, azole, echinocandin, and allylamine. For many years, the primary antifungal agent was amphotericin B, a polyene. This agent has a broad spectrum of activity in addition to fungicidal activity. Not only is this agent lethal to fungi, but it is also toxic

to humans. Patients treated with amphotericin B can experience many adverse side effects, including infusion-related reactions (fever, rigor, myalgia, and arthralgia) and renal impairment. Despite these problems, amphotericin B is frequently used for life-threatening fungal disease. Unfortunately, resistance to amphotericin B has been documented with *Scedosporium* spp., *L. prolificans*, *P. lilacinum*, *A. terreus*, and *Candida lusitanae* isolates.

The class that has provided the largest number of agents is the azoles. The most noteworthy compounds in this class include fluconazole, itraconazole, isavuconazole, posaconazole, and voriconazole. The azoles are important because they exhibit reasonable activity against fungi while causing fewer side effects. Fluconazole is the leading agent for treating yeast infections but has limited to no activity against molds. It is widely used by many practitioners to treat all types of infections, including vaginitis and thrush. Unfortunately, its overuse or misuse has resulted in the development of resistance, most notably with *C. glabrata*. Most *C. auris* isolates are also resistant to fluconazole, and *C. krusei* is considered intrinsically resistant. It is extremely important to determine the susceptibility pattern of a specific isolate before prescribing fluconazole for severe infections in patients who have been receiving long-term antifungal therapy.

The other azoles, itraconazole, isavuconazole, voriconazole, and posaconazole, are more frequently prescribed for treating mold infections. Itraconazole has been useful for treating aspergilli and phaeoid fungi infections. Isavuconazole and posaconazole have perhaps the broadest activity of the azoles against fungal species. Voriconazole has potent activity against the aspergilli, in addition to several emerging pathogens. Although prophylaxis with voriconazole is effective in decreasing infections by *Aspergillus* spp., it lacks activity against Mucorales.

The first agent to be released in the echinocandins group was caspofungin. This agent is lethal for yeast and, although effective against the aspergilli, is not generally lethal. This class targets cell wall synthesis, which makes it an attractive option for treating fungi that have developed resistance to other agents. Two other echinocandins are anidulafungin and micafungin. These two antifungals tend to have lower minimal inhibitory concentrations (MICs) compared with caspofungin. The CDC recommends an echinocandin for the treatment of invasive *C. auris* infections.

Two FDA-approved allylamines are terbinafine and naftifine. These compounds interfere with the synthesis of ergosterol, a principal sterol in the plasma membrane of many fungi. Terbinafine may be given orally or applied topically and is active against several groups of fungi, including the dermatophytes. Naftifine is used only topically.

Antifungal susceptibility testing

Until the past several years, many laboratories used methods that had been developed in-house to determine susceptibility patterns for the fungi. The Clinical and Laboratory Standards Institute (CLSI) in the United States has published four methods for antifungal susceptibility testing. These include M27 for yeast testing, M38 for mold testing, M44 for disk diffusion testing for yeasts, and M51 for disk diffusion testing for molds.

Although much debate exists regarding antifungal susceptibility testing, it is gaining popularity with more laboratories. The FDA has approved a microtiter method from TREK Diagnostic Systems (Thermo Fisher Scientific, Waltham, MA), a gradient diffusion method (Etest; bioMérieux), and MIC Test Strip (MTS; Liofilchem, Waltham, MA). Before these products were developed, only laboratories that could prepare their own reagents performed antifungal susceptibility testing.

The largest barrier to widespread antifungal testing is the lack of established breakpoints for most of the agents. Breakpoints are now species specific by drug, with results placing the organism categorically in the same manner as bacteria. These categories are S (susceptible), I (intermediate), and R (resistant) for the echinocandins. The azoles fluconazole and voriconazole are categorized as S, SDD (susceptible, dose dependent, fluconazole), I (for voriconazole), and R. The SDD category for fluconazole indicates isolates that may be considered susceptible when high-dose therapy, as opposed to standard therapy, is used.

Until breakpoints are established, the CLSI has provided epidemiologic cutoff values (ECVs). The ECV is an end point that can be used to evaluate a given MIC when clinical breakpoints are not available. The ECV does not indicate susceptible or resistant strains rather, wild-type and non-wild-type strains, where non-wild-type strains may harbor mechanisms of antifungal resistance. The health care provider will be able to review the activity of a drug against a specific strain to determine whether the MIC result is typical for that species or high. Those strains with an MIC above the ECV have potentially acquired mechanisms of resistance. Until other drugs are studied and breakpoints are established, the usefulness of antifungal susceptibility testing will most likely remain limited.

POINTS TO REMEMBER

- Yeasts typically reproduce by budding, whereas molds often reproduce by forming conidia/spores.
- A large array of fungi are capable of causing human infections.
- Fungi can be isolated from almost any type of clinical specimen.
- Fungal identification is based on a variety of characteristics, including macroscopic appearance, microscopic appearance, ability to grow at various temperatures, and biochemical reactions. Molecular and proteomic assays are now commonly used for fungal identification.
- Phaeoid (dematiaceous) fungi produce dark pigments.
- Dermatophytoses are caused by *Trichophyton*, *Microsporum*, and *Epidermophyton* species.
- The thermally dimorphic fungi *Blastomyces* spp., *Coccidioides immitis*, *Coccidioides posadasii*, *Histoplasma* spp., *Paracoccidioides* spp., *Sporothrix schenckii* species complex, and *Talaromyces marneffei* are often associated with systemic mycoses.
- Clinically important Mucorales include *Rhizopus*, *Mucor*, *Lichtheimia*, *Cunninghamella*, *Syncephalastrum*, *Apophysomyces*, and *Saksena*.
- *Geotrichum* is a mold that is often initially mistaken for a yeast.
- Saprobic fungi are most problematic in the immunocompromised host.
- *Candida albicans* is the most commonly isolated yeast and the one responsible for most human infections.
- *Pneumocystis jirovecii* is an important pathogen in patients with AIDS.
- Extreme care should be taken when working with dimorphic fungi in the laboratory. Cultures for all molds should be processed in a biological safety cabinet.
- Antifungal therapy is frequently ineffective when diagnosis is delayed.

LEARNING ASSESSMENT QUESTIONS

1. Which of the following organisms is the causative agent of tinea nigra?
 - a. *Microsporum canis*
 - b. *Trichosporon beigeli*
 - c. *Piedraia hortae*
 - d. *Hortaea werneckii*
2. Which of the following organisms is *not* a causative agent of chromoblastomycosis?
 - a. *Fonsecaea compacta*
 - b. *Talaromyces marneffei*
 - c. *Fonsecaea pedrosoi*
 - d. *Phialophora verrucosa*
3. A patient with very pale patches on his arms and legs is examined at his physician's office. His physician orders a fungal culture. The fungus shows a "spaghetti-and-meatball" appearance on direct smear. Which organism would you suspect?
 - a. *Microsporum canis*
 - b. *Malassezia furfur*
 - c. *Trichophyton rubrum*
 - d. *Epidermophyton floccosum*
4. Describe the characteristic microscopic appearance for each of the following dimorphic fungi when grown at 22° or 35°C.
 - a. *Blastomyces dermatitidis*
 - b. *Coccidioides immitis* and *C. posadasii*
 - c. *Histoplasma capsulatum*
 - d. *Sporothrix schenckii* species complex
5. What are the distinguishing microscopic characteristics for each of the following organisms?
 - a. *Nannizzia gypsea* (*Microsporum gypseum*)
 - b. *Microsporum canis*
 - c. *Trichophyton rubrum*
 - d. *Trichophyton mentagrophytes*
6. What are the expected results of the urease test and the hair perforation test for *T. rubrum* and *T. mentagrophytes*?
7. Which organism is one of the primary opportunistic infections in patients with acquired immunodeficiency syndrome?
 - a. *Pneumocystis jirovecii*
 - b. *Hortaea werneckii*
 - c. *Coccidioides immitis*
 - d. *Paracoccidioides brasiliensis*

8. What is the significance of isolating a saprobe from an infection in an immunocompromised patient?
9. How would you compare the macroscopic and microscopic morphology of the following saprobes: *Penicillium* spp., *Aspergillus fumigatus*, *Fusarium* spp., and *Curvularia* spp.?
10. What are the differences between chromoblastomycosis and eumycotic mycetoma?
11. What are the major differences between bacteria and fungi?
12. How are hyaline fungi different from phaeoid fungi?
13. Define *teleomorph*, *anamorph*, and *synanamorph*.
14. What are the expected results of the germ tube test for *Candida albicans*? What morphology would you likely see if you inoculated the colony onto cornmeal agar?
15. Large, one-celled, smooth to tuberculate macroconidia and smooth or echinulate microconidia are typical of the mycelial-phase growth of which of the following fungi?
 - a. *Blastomyces dermatitidis*
 - b. *Coccidioides immitis*
 - c. *Histoplasma capsulatum*
 - d. *Paracoccidioides brasiliensis*
16. Which of the following are the infectious structures of *Coccidioides immitis*?
 - a. Hyphae
 - b. Blastoconidia
 - c. Arthroconidia
 - d. Chlamydospores
17. Black-dot ringworm is an endothrix hair involvement caused by which of the following dermatophytes?
 - a. *Microsporum canis*
 - b. *Trichophyton tonsurans*
 - c. *Trichophyton mentagrophytes*
 - d. *Epidermophyton floccosum*
18. A subcutaneous fungal infection described as necrotic ulcers may follow direct inoculation of fungal spores into the skin. The causative agent when cultured at 37° C forms small, cigar-shaped yeasts and at room temperature, grows as a mold with delicate hyphae and rosette-like conidia. What is the name of this disease?
 - a. Blastomycosis
 - b. Chromomycosis
 - c. Sporotrichosis
 - d. Mycetoma
19. Most cases of pulmonary infections and invasive diseases caused by *Aspergillus* in humans result from which of the following organisms?
 - a. *Aspergillus fumigatus*
 - b. *Aspergillus flavus*
 - c. *Aspergillus niger*
 - d. *Aspergillus terreus*
20. A 10-year-old patient was admitted to the hospital with diabetic ketoacidosis. Three days later, he complained of nasal sinus blockage. Within 3 days, white fluffy mold grew from the sinus drainage and showed erect sporangioophores terminating in dark sporangia and sporangiospores. Brown rhizoids appeared at the base of the sporangioophores. Which of the following organisms would you suspect to identify?
 - a. *Alternaria*
 - b. *Mucor*
 - c. *Rhizopus*
 - d. *Curvularia*

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Diagnostic parasitology

Lauren Roberts and Connie R. Mahon

CHAPTER OUTLINE

General concepts in parasitology laboratory methods, 640

- Fecal specimens, 640
- Other specimens examined for intestinal and urogenital parasites, 643
- Examination of specimens for blood and tissue parasites, 644
- Immunologic diagnosis, 645
- Quality assurance in the parasitology laboratory 646

Medically important parasitic agents, 646

- Protozoa, 646
- Apicomplexa, 667
- Microsporidia, 681
- Helminths, 682
- Flukes, 682
- Tapeworms, 687
- Tissue infections with cestodes, 691
- Roundworms, 692
- Hookworms, 695
- Strongyloides stercoralis*, 697
- Blood and tissue roundworm infections, 700

Bibliography, 706

OBJECTIVES

After reading and studying this chapter, you should be able to:

1. List the major considerations in the collection and handling of specimens for the identification of intestinal and blood and tissue parasites.
2. Describe protocols for sample collection, handling, and transport of specimens for blood and tissue parasites.
3. Describe the general procedures for performing the direct wet mount, fecal concentration, and permanently stained smears.
4. Determine the stages of parasites found during microscopic examination of fecal material with direct wet mount, fecal concentration, and permanently stained smears.
5. Compare the procedures and uses of thick and thin blood smears for the identification of blood parasites.

6. Justify performing a thick and thin blood smear for the identification of blood parasites.
7. Justify the use of molecular assays to detect parasites in clinical specimens.
8. Compare the general characteristics of the major phyla of human parasites.
9. Discriminate between pathogenic and nonpathogenic protozoa on the basis of morphologic features.
10. Justify reporting nonpathogenic intestinal parasites when they are identified in clinical specimens.
11. For the major human pathogens, describe the mechanism of pathogenesis, clinical symptoms, treatment, and prevention.
12. For each organism presented, describe the morphology; life cycle, including the infective and diagnostic stages; and usual procedure for identification.

KEY TERMS

Amastigote	Hexacanth embryo (oncosphere)
Appiqué forms	Intermediate hosts
Asexual reproduction	Karyosome
Axostyle	Kinetoplast
Blackwater fever	Maurer dots
Bradyzoites	Merozoites
Cercaria	Metacercaria
Chromatoidal bars	Microfilariae
Cyst	Miracidium
Cysticercus	Peripheral chromatin
Cytostome	Polyvinyl alcohol (PVA)
Definitive hosts	Primary amebic meningoencephalitis (PAM)
Erythrocytic phase	Proglottids
Exoerythrocytic phase	Rapid diagnostic tests (RDTs)
Filariform larvae	Rhabditiform larva
Gametes	Schizogony
Gametocytes	Schizont
Granulomatous amebic encephalitis (GAE)	Schüffner stippling

Scolex
Sexual reproduction
Sporoblast
Sporocysts
Sporogony
Sporozoites

Tachyzoites
Trophozoites
Trypomastigote
Undulating membrane
Vectors
Ziemann dots

Case in point

A 4-year-old male patient was brought to the public health clinic because of intermittent bouts of diarrhea lasting almost 4 weeks. The mother did not note any bright red blood in his stool. The child was pale, listless, and had a protuberant abdomen. He had several small erythematous vesicles on his feet. His mother said that he sometimes ate dirt and always had a good appetite. The family lived in a rural part of Georgia and had a well from which they obtained their drinking water. This part of the county had only recently been connected to the local city's sanitation system. The white blood cell (WBC) count was $10.2 \times 10^3/\mu\text{L}$ (reference range, 4.8×10^3 to $10.8 \times 10^3/\mu\text{L}$), and the differential showed 14% eosinophils (reference range, 0% to 4%). The hemoglobin level was 6.2 g/dL (reference range, 9 to 14 g/dL), and the reticulocyte count was 8% (reference range, 0.5% to 2%). The red blood cell (RBC) morphology was described as microcytic and hypochromic. The physician ordered a stool culture for bacterial pathogens and an ova and parasite (O & P) examination. The bacterial culture was negative for enteric pathogens, but the O & P examination revealed parasitic organisms and the presence of Charcot-Leyden crystals.

Issues to consider

After reading the patient's case history, consider:

- Significant features in the patient's history
- Intestinal parasites that should be considered part of the patient's differential diagnosis
- Additional laboratory tests that could aid in the diagnosis

Parasites are an important cause of human morbidity and death in many parts of the world. However, in the United States and other developed countries, they are seldom regarded as major causes of disease. Although parasites are often associated with gastrointestinal (GI) infection or, in the case of malaria, a blood infection, other parasites can live in organs and be transmitted via organ transplants. Some can invade the central nervous system (CNS). A few can cross the placenta and cause congenital infection. Health care professionals have become increasingly aware that parasites should be considered as possible causative agents of a patient's clinical condition. The factors that have led to this greater awareness include the rising number of immunocompromised patients susceptible to infections caused by known pathogens and opportunistic organisms, the increase in the number of people who travel to countries that have less than ideal sanitation and a large number of endemic parasites, and the growing population of immigrants from areas with endemic parasites. In addition, there is concern that ongoing climate change might affect the movement of insect vectors into new geographic areas, thus expanding the range of some diseases normally associated with tropical climates.

When a health care provider encounters a case of an infection that may be caused by a parasite, the patient's symptoms and clinical history, including travel history, are significant

data that must be gathered and shared with the laboratory scientist. The clinician and laboratory scientist should collaborate to make sure that the appropriate specimen is properly collected, handled, and examined. Parasitic infections can be difficult to diagnose, however, because patients often have nonspecific clinical symptoms that can be attributed to a number of disease agents. Knowledge of common pathogens and nonpathogens that exist in specific geographic regions and for a given body site is necessary to ensure proper identification and, if necessary, therapy.

Detection and identification of a parasite depend not only on the adequacy of the submitted specimen but also on the procedures established by the clinical laboratory, including the criteria for specimen collection, handling, and transport and for the laboratory methods used. Due to the low incidence of parasitic infections in the United States, many laboratories do not have extensive experience detecting parasites. This chapter discusses the major medically important parasites, their epidemiology and life cycle, and the clinical infections they cause. In addition, it presents the diagnostic features that characterize these agents. Readers are referred to parasitology references for detailed procedures, reagent preparation, and a comprehensive description of parasites that have been implicated in human disease.

General concepts in parasitology laboratory methods

Fecal specimens

Collection, handling, and transport

The collection and handling of a stool specimen before laboratory examination may influence whether organisms will be identified. The stool should be delivered to the laboratory as soon as possible after collection, or a portion should be placed immediately in a preservative. **Trophozoites, the motile and reproductive form of some amebae, or eggs of some helminths may disintegrate if not preserved or examined within a short time after collection.** Because many intestinal organisms are shed into the stool irregularly, a single stool specimen may be insufficient to detect an intestinal parasite. Studies have shown that only 58% to 72% of protozoa are detected with a single specimen. Traditionally, for optimal detection of intestinal parasites, a series of **three stool specimens collected a day or two apart, within a 10-day period,** has been recommended. This procedure of examining each specimen submitted is time-consuming and labor-intensive. Published articles suggest that in some circumstances, pooling of the three formalin-preserved specimens gives a parasite recovery rate comparable with that of the individual examination of formalin-preserved stools. Although this method saves time, there is a risk that if organisms are present in small numbers, they may be missed on microscopic examination of wet mounts because of a dilution effect (i.e., reduced sensitivity). Another cost-effective option includes examination of a second or third specimen only if the first test is negative and the patient remains symptomatic. Many laboratories establish an algorithm that considers the types of parasitology examinations routinely performed (e.g., antigen detection, nucleic acid

amplification testing [NAAT], fecal concentration, permanently stained smear, patient population, and specific criteria (e.g., travel, symptoms, immune status, inpatient or outpatient classification) to determine if a single specimen or multiple stool specimens should be examined.

It has been recommended that permanently stained smears be made and examined for each specimen individually. In addition, whether the patient is an inpatient or outpatient, the presence of symptoms should dictate whether three specimens are needed. For example, although inpatients frequently acquire healthcare-associated bacterial infections, it is highly unlikely that an inpatient would acquire a healthcare-associated parasitic infection. An immunoassay for *Giardia duodenalis* or *Cryptosporidium* may be requested on specimens from immunocompromised patients with diarrhea or children in daycare settings who are symptomatic in place of, or in addition to, the usual O & P procedure. Reflex testing often begins with an immunoassay or multiplex procedure for parasite antigens of the more common organisms, such as *Giardia*, and then a routine O & P examination is performed, if the results are negative, and the patient is symptomatic.

The collection container for feces should be clean, dry, sealed tightly, and waterproof (e.g., a plastic container with lid). Commercial systems that incorporate collection container and preservatives are also available. Stool specimens should never be collected from bedpans or toilet bowls; such practice might contaminate the specimen with urine or water, resulting in the destruction of trophozoites or introduction of free-living protozoa. As an alternative, the specimen could be collected on a clean piece of waxed paper or newspaper and transferred to the container. Another alternative is to use a disposable collection container that can be fitted under the toilet bowl rim. The specimen should be submitted as soon as possible after passage. The specimen should be properly labeled as discussed in Chapter 6.

Stool specimens for parasites should be collected before a barium enema, certain procedures using dyes, or the start of antimicrobial therapy. Antimicrobials can reduce the number of organisms present. If the patient has undergone a barium enema, stool examination should be delayed for 7 to 10 days because barium obscures organisms when specimens are examined microscopically, even after concentration procedures. If a purged specimen is to be collected, it is recommended that a saline or phosphosoda purgative be used because mineral oil droplets interfere with identification of parasites, especially protozoan cysts, an infective dormant form resistant to environmental stress. The second or third specimen after the purge is more likely to contain trophozoites that inhabit the cecum.

Preservation

Several methods are available for stool preservation if the specimen will not be delivered immediately to the laboratory. The preservative used is determined by the procedure to be performed on the fecal sample. Regardless of the preservative used, the ratio of three parts preservative to one part feces should be maintained for optimal fixation. Table 28.1 presents some of the more common preservatives and their appropriate use. The time that the stool was passed and the time it was placed in the fixative should be noted on the laboratory requisition and container. A commercially available two-vial system using modified polyvinyl alcohol (PVA), a resin polymer, in

Table 28.1 Preservatives commonly used for fecal samples

Preservative	Laboratory examination method
Modified polyvinyl alcohol	Permanently stained smear, DNA-PCR
10% formalin	Formalin–ethyl acetate concentration, direct wet mount, and most immunoassays
Sodium acetate–acetic acid–formalin	Permanently stained smears, concentration, and most immunoassays
Merthiolate–iodine–formalin	Concentration and direct wet mount
Single-vial systems (EcoFix, Parasafe, PROTO-FIX)	Concentration, direct wet mount, permanently stained smears, and most immunoassays

DNA, Deoxyribonucleic acid; PCR, polymerase chain reaction.

one vial and 10% formalin in the other vial is commonly used. The system comes with patient instructions and a self-sealing plastic bag for transport. The classic PVA fixative, which consists of mercuric chloride (for fixation) and PVA (to increase adhesion of the stool to the slide), was traditionally used when a permanently stained smear was to be made. Because of the toxicity of mercuric chloride, modifications that do not contain mercury have been developed. The modified PVA preservatives do not provide the same level of microscopic detail as the mercury-based formula, but they are safer acceptable alternatives. Schaudinn fixative also contains mercuric chloride. The formalin vial is used for direct wet mount and concentration procedures.

Alternative nontoxic fixatives, EcoFix (Meridian Bioscience, Cincinnati, OH), Parasafe (Scientific Device Laboratory, Des Plaines, IL), and PROTO-FIX (Alpha-Tec Systems, Vancouver, WA) are single-vial fixatives for wet mounts and permanently stained slides that do not use formaldehyde or mercury compounds. The specimen only needs to be added to one vial that can be used for wet mount, concentration, and permanently stained slides. Evaluation of stains made from stools preserved with these compounds has shown differing quality of the stained preparations. In some cases, the background quality was poor, and in others a less sharp morphology of the organism was observed. The fixative sodium acetate–acetic acid–formalin (SAF) is another alternative that can be used for the preservation of fecal specimens when concentration procedures and permanent stains will be used.

Macroscopic examination

The examination of an unpreserved stool specimen should include macroscopic (gross) and microscopic procedures. The initial laboratory procedure is the macroscopic examination. Laboratories that receive stool specimens already placed into preservative(s) are not able to perform a macroscopic examination. During gross examination, intact worms or proglottids (tapeworm segments) can be seen on the surface of the stool. Gross examination of the specimen also reveals the consistency (liquid, soft, formed) of the stool sample. Consistency may help determine the type of preservative to be used, indicate the forms of parasites expected to be present, or dictate the immediacy of examination. Fig. 28.1 shows the relationship between stool consistency and protozoan stage. Cysts (infective stage) are most likely to be found in formed stool

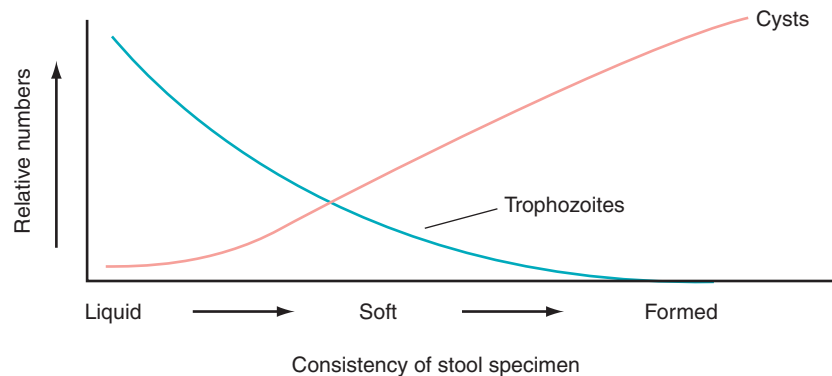


Fig. 28.1 Relationship of stool consistency to protozoan stage.

and sometimes in soft stool, whereas liquid stool will most likely contain the trophozoite stage.

During gross examination, the color of the stool specimen is noted. A normal stool sample usually appears brown. Stool that appears black may indicate **bleeding in the upper GI tract**, whereas the presence of fresh blood may indicate bleeding in the lower portion of the intestinal tract. Any portion of the stool that contains blood or blood-tinged mucus should be selected for wet mount preparations and added to preservative. A liquid stool specimen should be examined within 30 minutes of passage to detect the presence of motile trophozoites. A formed stool specimen should be examined within 2 to 3 hours of passage if held at room temperature; however, examination may be delayed up to 24 hours after passage if the specimen is placed at 4° C. The specimen should not be placed in a 37° C incubator, which increases the rate of disintegration of any organisms present and enhances overgrowth by bacteria. Following macroscopic examination, the specimen should be placed into the appropriate fixative(s) for concentration and preparation of permanently stained smear.

Microscopic examination

Several diagnostic methods can be used in the microscopic examination of a fecal specimen:

- Direct wet mount examination (iodine-stained and unstained) of fresh stool specimens
- Concentration procedures with wet mount examination of the concentrate
- Preparation of permanently stained smears

In general, concentration and permanent staining procedures should be performed on all specimens.

Wet mount preparations

The direct wet mount of **unpreserved fecal material is primarily used to detect the presence of motile protozoan trophozoites in a fresh liquid stool or from sigmoidoscopy material.**

A liquid stool specimen or purged specimen may contain motile protozoan trophozoites; hence, purged specimens should be examined immediately (within 30 minutes) after passage to ensure motility of the organisms. A portion should also be placed in a fixative so permanently stained smears for definitive identification can be made. Formed stools are unlikely to yield motile trophozoites, thus a direct wet mount

examination is not necessary. Direct wet mounts are not performed on specimens received in fixatives.

The wet mount procedure uses a glass slide on which a drop of physiologic saline (0.85%) has been placed at one end and a drop of iodine (Dobell and O'Connor iodine, D'Antoni iodine, or 1:5 dilution of Lugol solution) at the other end. A small amount (2 mg) of feces is added to each drop and mixed well. Each preparation should be covered with a No. 1, 22-mm square coverslip. The preparation should be thin enough so newsprint can be read through it and should not overflow beyond the edges of the coverslip. In unfixed stool specimens, the saline preparation is useful for the detection of helminth eggs or larvae, motile protozoa, and refractile protozoan cysts. Iodine emphasizes nuclear detail and glycogen masses but kills trophozoites. Wet mounts are also made from a fecal specimen following a concentration procedure. This detects protozoan cysts, helminth eggs, and helminth larvae.

Reading the wet mount involves thorough examination of each coverslipped preparation at low power, starting at one corner and following a systematic vertical or horizontal pattern until the entire preparation has been examined. A high-power objective is used to identify any suspicious structures. Oil immersion should not be used on a wet preparation unless the preparation has been sealed.

Concentration techniques

Concentration techniques are designed to concentrate the parasites present into a small volume of fluid and remove as much debris as possible. Fresh or stool specimens in an acceptable preservative may be used. The concentrate sediment may then be examined unstained or stained with iodine. Protozoan trophozoites do not survive the procedure. Protozoan cysts, helminth larvae, and helminth eggs, however, can be detected using this method.

Sedimentation and flotation methods, both of which are based on the difference in specific gravity between the parasites and concentrating solution, are used to concentrate parasites into a small volume for easier detection and increased sensitivity. In sedimentation methods, the organisms are concentrated in sediment at the bottom of the centrifuge tube. In flotation methods, the organisms are suspended at the top of a high-density fluid. Overall, sedimentation methods concentrate a greater diversity of organisms, including cysts, larvae, and eggs. The formalin-ethyl acetate sedimentation (FES) method was the standard sedimentation method. A number of manufacturers now market self-contained fecal

concentration kits that do not use the solvent ethyl acetate. Some kits incorporating collection and concentration in a single tube may not require centrifugation, making them useful in rural areas and developing countries. Although these kits offer easier disposability and a cleaner preparation because of the filtration method used, studies indicate that their use is more expensive than that of the traditional FES method.

The zinc sulfate method is the usual flotation procedure, although other methods such as zinc chloride are also available. While the flotation methods yield less fecal debris in the finished preparation compared with the FES method, the flotation liquids can cause operculated eggs to open or collapse. It also tends to distort protozoan cysts. When used, this procedure may miss infertile *Ascaris lumbricoides* eggs and *Schistosoma* spp. eggs. Because of their high density, these eggs sink to the bottom of the test tube. Most organisms tend to settle after about 30 minutes. Therefore the examination should be performed as soon as possible after the procedure has been completed to ensure optimal recovery of organisms.

Permanently stained smears

Permanently stained smear preparations of all stool specimens should be made to detect and identify protozoan trophozoites and cysts. The characteristics needed for identification of the protozoa, including nuclear detail, size, and internal structures, are visible in a well-made and properly stained smear. The permanent stains commonly used include iron hematoxylin and trichrome (Wheatley modification of the Gomori stain). The stain of choice in most laboratories is the trichrome stain because results are somewhat less dependent on the technique, and the procedure is less time-consuming. Although some laboratories prepare the stain in-house, manufacturers provide prepared stains and reagents for this procedure. Trichrome staining can be performed on a smear made from a fresh stool specimen fixed in Schaudinn fixative or from one that has been preserved in PVA. Specimens preserved in SAF do not stain well with trichrome and should be stained with iron hematoxylin.

To prepare a trichrome-stained smear of a fresh specimen, applicator sticks are used to smear a thin film of stool across a 1- by 3-inch slide. The smear is placed immediately in Schaudinn fixative; it must not be allowed to dry before fixation. For PVA-fixed specimens, several drops of specimen are placed on a paper towel to drain excess fluid. The material on the paper towel is collected to prepare the smear in the same way as for a fresh specimen. The specimen should be allowed to air-dry thoroughly before staining.

In a well-stained trichrome smear, the cytoplasm of protozoan cysts and trophozoites stains blue-green, although *Entamoeba coli* often stains purple. Nuclear peripheral chromatin (deoxyribonucleic acid [DNA] and proteins on the edge of the nucleus), karyosome (mass of chromatin in the nucleus), chromatoidal bars (dark-staining cytoplasmic inclusions of chromatin), and RBCs stain dark red to purple. Eggs and larvae stain red; however, they are often distorted or destroyed in the staining process. Background debris and yeasts stain green. With an iron hematoxylin stain, the parasites stain gray to black, nuclear material stains black, and background material stains light blue to gray. With either stain, poor fixation of fecal material results in poorly staining or nonstaining organisms. Several modifications of the trichrome staining procedure have been developed to allow the detection of a group of organisms known as the *microsporidia*.

All permanently stained smears should be examined by first scanning for thick and thin areas using lower-power magnification ($\times 10$ or $\times 40$ objective). Thin areas should be selected and examined under oil immersion ($\times 100$ objective) for identification of organisms. It should take approximately 10 to 15 minutes to adequately examine selected areas. Organisms that stain lightly and may be difficult to identify in either the cyst or the trophozoite stage include *Entamoeba hartmanni*, *Dientamoeba fragilis*, *Endolimax nana*, *Chilomastix mesnili*, and *Giardia duodenalis*.

Modified acid-fast stain

The Kinyoun modified acid-fast stain is used to detect oocysts of *Cryptosporidium* spp., *Cystoisospora belli* (formerly *Isospora belli*), and *Cyclospora cayetanensis*. With this procedure, the oocysts appear as magenta-stained organisms against a blue background.

Other specimens examined for intestinal and urogenital parasites

Cellophane tape preparation for pinworm

The life cycle of the pinworm *Enterobius vermicularis* includes migration of the female from the anus at night to lay eggs in the perianal area. Therefore a fecal specimen is not the optimal specimen for diagnosis of infection with this organism. Instead, the cellophane tape preparation is routinely used for detection of suspected pinworm infections. This procedure involves swabbing the person's perianal area with a tongue blade covered with cellophane tape (sticky side out). The collection should take place first thing in the morning before the individual uses the bathroom or has bathed. After the sample has been taken, the sticky side of the tape is placed on a microscope slide and scanned at low- and high-power magnification for the characteristically shaped eggs. Adaptations of this procedure using paddles with a sticky surface are commercially available.

Duodenal aspirates

Material obtained from duodenal aspirates or from the duodenal capsule Entero-Test (HDC, San Jose, CA) may be submitted in cases of suspected giardiasis or strongyloidiasis when clinical symptoms are suggestive of infection but repeated routine stool examination results are negative. In the Entero-Test, the patient swallows a gelatin capsule containing a weighted string. One end of the string is taped to the side of the patient's mouth; the weighted end is carried into the upper small intestine. After about 4 hours, the string is brought up, and part of the mucus adhering to the surface is stripped off and examined on a wet mount for motile trophozoites. The remainder of the specimen is placed in a fixative for a permanently stained smear. Eggs of *Fasciola hepatica* and *Clonorchis sinensis* as well as oocysts of *Cryptosporidium* and *C. belli* can also be recovered.

Sigmoidoscopy specimens

Scrapings or aspirates obtained by sigmoidoscopy may be used to diagnose amebiasis or cryptosporidiosis. These specimens are examined immediately for motile trophozoites, and a portion of the sample is placed in PVA fixative so

permanently stained smears can be prepared for examination. If cryptosporidiosis is suspected, a smear for staining with a modified acid-fast stain or by a fluorescent procedure should be prepared.

Urine, vaginal, and urethral specimens

Eggs of *Schistosoma haematobium* and *E. vermicularis* and trophozoites of *Trichomonas vaginalis* can be detected in urine sediment. *T. vaginalis* can also be detected in a wet mount of vaginal or urethral discharge. Envelope culture methods for *T. vaginalis*, such as the InPouch TV (Biomed Diagnostics, White City, OR), are also available. In this system, the specimen is added to the medium and incubated. Growth of the organism can be microscopically observed through the pouch within 3 days. Rapid antigen detection kits and molecular assays for *T. vaginalis* are also available.

Sputum

In cases of *Strongyloides stercoralis* hyperinfection, **filariiform larvae** may be seen in a direct wet mount of sputum and bronchoalveolar lavage and washings. Larval forms of other migrating helminths, such as *A. lumbricoides* and hookworm, can also be detected. Eggs of the lung fluke *Paragonimus westermani* can be identified in a wet mount. In addition, *Entamoeba histolytica*, *Cryptosporidium* oocysts, and microsporidia can be found in lower respiratory tract specimens. If the patient is suspected of having a pulmonary infection caused by these agents, the specimen should be examined as a permanently stained smear.

Examination of specimens for blood and tissue parasites

Blood smears

Depending on the blood parasite, whole blood or buffy coat preparations can be used. Examination of a whole blood smear stained with the Giemsa or Wright stain is the most common method of detecting *Plasmodium*, *Babesia*, *Trypanosoma*, and some species of microfilaria. Although motile organisms, such as *Trypanosoma* trypomastigotes and **microfilariae** (larval form of filarial worms), can be detected on a wet preparation of a fresh blood specimen under low- and high-power magnification, identification is made on the basis of characteristics seen on a permanently stained smear. Stained smears from buffy coats are helpful in finding *Leishmania* spp. and *Toxoplasma gondii* in the cytoplasm of mononuclear cells. The yeast form of the dimorphic fungus *Histoplasma capsulatum* can also be seen. Concentration methods using membrane filters can be used to detect *Trypanosoma* spp. or microfilariae, but these methods are rarely used in the clinical laboratory.

Collection and preparation

Blood taken directly from a finger stick is the ideal specimen for a malarial smear because it tends to give the best staining characteristics. Blood collected in ethylenediamine tetraacetic acid (EDTA) gives adequate staining if processed within 1 hour. Distortion of the organism may occur if the time to preparation of the slide is longer than 1 hour, and organisms may be

lost if the time exceeds 4 hours. For example, the **gametocytes** of *Plasmodium falciparum* may lose their characteristic banana shape and round up. With Giemsa stain, the cytoplasm of the parasite stains bluish, and the chromatin stains red to purple red. If malarial stippling is present, it appears as discrete pink-red dots. The Giemsa stain gives the best morphologic detail but is a time-consuming procedure. Wright stain has a shorter staining period, but the color intensity for the differentiation of parasites is not as good as that with Giemsa stain.

Identification procedure

For suspected cases of blood parasites, a thick film and a thin film should be made. Both preparations can be made on the same slide or on separate slides. Because the two preparations are treated differently before staining, however, use of two slides may be more convenient. The Giemsa stain provides the best staining of the organisms and should be used on thick and thin films. Thin smears must first be fixed in methanol before staining with the Giemsa stain. RBCs in the unfixed thick smear will lyse during the staining procedure. Unless the RBCs on the slide are first lysed in distilled water, Wright stain cannot be used for a thick film because the stain contains methanol, which will fix RBCs.

A thick film is best for the detection of parasites (high sensitivity) because of the larger volume of blood and the fact that organisms are concentrated in a relatively small area. The thick film is made by pooling several drops of blood on the slide and then spread into a circular area with a diameter approximately equal to 1.5 cm. A film that is too thick peels from the slide; thickness is optimal when newsprint is barely visible through the drop of blood before it dries. The blood should be allowed to dry for at least 6 hours before staining. It should not be fixed with methanol before staining; fixing prevents lysis of the RBCs. The Giemsa stain releases hemoglobin by lysing unfixed RBCs. Initial scanning of the stained smear at $\times 100$ magnification detects microfilariae. The thick smear is examined at $\times 1000$ magnification for the presence of malarial organisms. In the thick film, the RBCs are destroyed, so only WBCs, platelets, and parasites are visible. In a thick film, the organisms may be difficult to identify, and there is no way to compare the size of infected and noninfected erythrocytes. Therefore species identification should be made from a thin film because the characteristics of the parasite and the RBCs can be seen.

The thin film is made in the same way as that for a differential cell count. It should be fixed in methanol for 1 minute and air-dried before staining with Giemsa stain. The entire smear should be scanned at low-power ($\times 100$ magnification) for detection of large organisms such as microfilariae; then at least 100 oil immersion fields ($\times 1000$ magnification) must be examined for the presence of organisms such as *Trypanosoma* or intracellular organisms such as *Plasmodium* or *Babesia*. Because the RBCs are intact, the thin smear has a higher specificity compared with a thick smear. For a symptomatic patient, several blood smears from samples collected at approximately 6-hour intervals over 36 to 48 hours should be examined before a final negative diagnosis is made. *Parasitemia* (percentage of erythrocytes parasitized) can be calculated from the thin blood smear. It is recommended that a minimum of 500 RBCs be counted. The calculation is as follows:

$$\left(\frac{\text{Number of infected RBCs}}{\text{Total number of RBCs counted}} \right) \times 100 = \text{Percent infected RBCs}$$

Biopsy specimens

Biopsy specimens are typically examined using stained tissue sections processed in the histology section of a clinical laboratory. Tissues that might yield parasites include skin, muscle, cornea, intestine, liver, lung, and brain. Biopsy specimens are usually needed to diagnose infections with *Leishmania* spp. because the organisms are intracellular. Depending on the species present, the amastigote (obligate intracellular form) can be detected in tissues, such as skin, liver, spleen, and bone marrow. Cutaneous lesions should be sampled below the edges of the ulcer; surface samples do not yield infected cells. Biopsy of striated muscle may also be performed for the diagnosis of *Trichinella spiralis*. *T. gondii* can also be identified by examination of tissue and muscle biopsy specimens.

Cerebrospinal fluid

Viable organisms in suspected cases of amebic meningitis or sleeping sickness can occasionally be seen in a cerebrospinal fluid (CSF) specimen. The **trypomastigote**, an extracellular nonreplicating form, is readily visible because of the motion of the flagellum and undulating membrane. It requires a skillful microscopist, however, to discern amebic motility in a field of neutrophils. If amebic meningoencephalitis caused by *Naegleria fowleri* is suspected, CSF can be cultured. Nonnutrient agar is seeded with an *Escherichia coli* overlay, and the CSF sediment is inoculated onto the medium. The specimen is sealed and incubated at 35° C. The medium is microscopically examined daily for thin tracks made by the amebae as they feed on the bacteria.

Immunologic diagnosis

Parasites that invade tissue (e.g., *E. histolytica*, *Trypanosoma cruzi*, or *T. gondii*) are the primary organisms that stimulate antibody production. Serologic tests are sometimes useful if invasive methods cannot be used for identification. In most cases, however, tests for antibody serve only as epidemiologic markers, especially in endemic areas. Detection of immunoglobulin M (IgM) can be useful in identifying infection during the acute phase, but this class of antibody generally declines to nondetectable levels as the infection begins to resolve. Detection of immunoglobulin G (IgG) does not distinguish between a relatively recent infection and a past infection because this class of antibody can persist for years. In some cases, however, detection of antibody is useful—for example, a patient who lives in an area nonendemic for a parasite has recently traveled to an endemic area and now shows symptoms, but the organism has not been detected in a clinical specimen. A positive test for antibody would help confirm a diagnosis. Another disadvantage of antibody tests is that they can have many cross-reactions that limit their diagnostic usefulness. In addition, it is difficult to obtain quality parasite antigen. Many serologic tests are used by reference laboratories, such as the U.S. Centers for Disease Control and Prevention (CDC), and are not commercially available.

The parameters that should be considered in selecting a method to be used should therefore include not only cost but also diagnostic yield, patient population, relative incidence of the parasite in the area, and number of specimens to be processed. Classically, such methods as hemagglutination or complement fixation were used to detect antibodies to

parasitic organisms. Newer tests use fluorescent or enzyme immunoassay (EIA) techniques. Immunoassay tests for antibodies to *T. gondii* or *E. histolytica* (extraintestinal infections) are available for use in clinical laboratories.

Enzyme immunoassays

The major use of EIA has been to detect parasite antigens in clinical specimens. These tests, in contrast with antibody tests, provide information about current infection. Several EIAs are available to detect the presence of *Giardia duodenalis*, *Cryptosporidium* spp., *E. histolytica*, and *Entamoeba dispar*. The sensitivity and specificity of these tests to detect intestinal parasites is excellent compared to microscopic examination. Some can find more than one parasite in the specimen by detecting antigens of the organism in the stool using organism-specific antibodies immobilized on a membrane. Rapid EIA kits that can be used with fresh or preserved fecal specimens and kits using monoclonal antibody to detect *Giardia* and *Cryptosporidium* antigens are also available. Not all kits detecting *E. histolytica*, however, can differentiate between *E. histolytica* (pathogenic) and *E. dispar* (nonpathogenic). Antigen detection kits have replaced microscopic examinations in some hospital laboratories.

Another growing area of EIA application testing is field diagnosis of malaria. Although most of these tests are used in endemic areas, one has been approved by the U.S. Food and Drug Administration (FDA) for use in the United States. These tests are based on the principle of immunochromatographic antigen capture (see [Chapter 10](#)), and use whole blood to detect malarial proteins. The patient's blood is reacted with monoclonal antibody labeled with dye or gold particles. Some tests are relatively non-species specific and detect a protein, such as parasite lactate dehydrogenase or aldolase, that is common to all four human *Plasmodium* spp. Other tests may detect a species-specific protein, such as histidine-rich protein, which is associated with *P. falciparum*. Some tests detect both types of protein to provide a more complete picture of the infective agent.

Fluorescent antibody techniques

Direct fluorescent antibody (DFA) techniques using monoclonal antibodies have been developed to detect *Cryptosporidium* oocysts in fecal specimens. Fecal material is spread on a slide, reagent containing the antibody is added, and the specimen is examined under a fluorescent microscope for a characteristic apple-green structure. These methods are more expensive than the modified acid-fast procedure but demonstrate greater sensitivity, especially when only rare oocysts are present. A DFA combination reagent for *G. duodenalis* and *Cryptosporidium* antigens is also available. The monoclonal antibodies help avoid false-positive and false-negative results. Such procedures are useful in screening large numbers of specimens during epidemiologic studies. DFA assays have greater sensitivity and specificity compared with routine stains.

The quantitative buffy coat (QBC; Drucker Diagnostics, Port Matilda, PA) procedure uses the fluorescent dye acridine orange to stain nuclear material for detecting malarial organisms in blood. A microhematocrit tube is coated with the fluorescent dye. After centrifugation, the parasites can be viewed

in a small area at the top of the erythrocyte column. This method is more sensitive than a thick smear in demonstrating the presence of parasites, but a thin smear must still be made for definitive identification. However, the instrumentation required may not be available for use in all areas of the world.

Molecular methods

Molecular methods for parasite identification are rapidly being adopted by clinical laboratories. Methods can include classic and real-time polymerase chain reaction (PCR) assays. Molecular methods of detection exist that identify several organisms, including malarial parasites. Multiplex PCR panels are commercially available for GI pathogens that can detect a significant number of viruses and bacteria and a limited number of parasites (e.g., *Giardia duodenalis*, *E. histolytica*, *Cryptosporidium* spp., and in some cases *Cyclospora cayentanensis*). *Dientamoeba fragilis*, *Blastocystis* spp., and microsporidia do not yet have a rapid identification procedure. Several of the current molecular procedures are rapid and do not require specimen processing. Although these newer methods have eliminated the need for routine concentration for selected organisms, the downside is that if the test result is negative and the patient remains symptomatic, routine procedures then should be instituted.

Quality assurance in the parasitology laboratory

Quality assurance procedures in the parasitology laboratory are like those in other laboratory sections. An updated procedure manual, controls for staining procedures, and records of centrifuge calibration, ocular micrometer calibration, and refrigerator and incubator temperatures should be available. Reagents, solutions, and kits must be properly labeled. In addition, the parasitology laboratory should have the following:

- A reference book collection, including texts and atlases
- A set of digital images of common parasites; many are available online
- A set of clinical reference specimens, including permanently stained smears and formalin-preserved feces

The department should also be enrolled in an external proficiency testing program. An ongoing internal proficiency testing program can be used to enhance the identification skills of the clinical laboratory scientists, especially if a full-time parasitologist is not employed. It has been shown that approximately twice as many parasites are detected when a single laboratory scientist staffs the parasitology department compared with departments that rotate personnel through the department. One type of program might assess the reproducibility of results in the examination of fecal specimens. Preserved specimens that have been reported are reexamined as part of this program to see if the initial results (organism identification and quantification) are duplicated.

Size is an important diagnostic criterion for parasites, and use of a properly calibrated ocular micrometer ensures accurate measurement of organisms. The micrometer should be calibrated for each objective on the microscope. Calibration requires two parts: (1) the stage micrometer, a 0.1-mm line

that is ruled in 0.01-mm units, and (2) the ocular micrometer, which is ruled in 100 units but has no value assigned to the units. Values for each ocular unit can be calculated by using the stage micrometer according to the procedure found in [Appendix D](#) on Evolve.

Medically important parasitic agents

Historically, the human medically important eucaryotic parasites were classified as protozoan, helminth, and arthropod. More recently the microsporidia, which belong to the kingdom of fungi, were recognized as human pathogens. Due to their morphology and pathogenesis, the microsporidia are often considered with the parasites. Medically important parasites can be found in phyla representing single-celled organisms, such as the protozoa and sporozoa, and complex, multicelled organisms, such as the helminths (worms). [Table 28.2](#) lists the characteristics of the phyla in which most medically important human parasites are found; they are described in this chapter. The protozoan parasites are classified according to their motility organelles; the ameba move by pseudopodia, the flagellates move by flagella, and the ciliates move by cilia. The sporozoa are nonmotile. Helminths are classified according to their shape and include flatworms (flukes and tapeworms) and roundworms. Arthropods, such as *Pediculus humanus* (pediculosis) and *Sarcoptes scabiei* (scabies), cause human infestations. They can also sometimes be vectors for infectious pathogens. These agents are discussed in [Chapter 33](#).

Protozoa

The protozoan parasites include pathogens and nonpathogens (commensals). Some of the commensal organisms closely resemble the pathogens, so it is important for the laboratory scientist to be able to recognize and distinguish among them. This ensures that a patient will be given appropriate therapy when necessary and not be treated unnecessarily. Commensal parasites must be reported in the test results. It is important to recognize that the commensals are not considered normal biota, but their presence indicates that the patient has ingested fecally contaminated food or water. When a commensal is detected, it is important for the laboratory scientist to examine the specimen closely for the presence of a pathogen.

Intestinal amebae

In general, amebae present the most difficult challenge regarding identification. Their average size range is smaller than that of most other parasitic organisms, and they must be distinguished from artifacts and cells that appear in the clinical specimen. Species identification, whether in the cyst or in the trophozoite stage, often is based on the size, number of nuclei, nuclear structure, and presence of specific internal structures. In a direct wet preparation, the motility of the trophozoite may aid in presumptive identification. Overall, however, the permanently stained smear is the best preparation for identification of the amebae.

E. histolytica is recognized as a true pathogen, whereas *Blastocystis* spp. still have questionable status as a pathogen. Other amebae are considered nonpathogens. All of the amebic

Table 28.2 Characteristics of phyla of medically important parasites

Organisms	Characteristics
Phylum: Sarcomastigophora	
Subphylum: Sarcodina (ameba)	Single celled Move by pseudopodia Trophozoite and cyst stages Asexual reproduction
Subphylum: Mastigophora (flagellates)	Single celled Most move by action of flagella Trophozoite and cyst stages for intestinal organisms, except for <i>Dientamoeba fragilis</i> Asexual reproduction Some blood flagellates
Phylum: Ciliophora (ciliates)	Single celled Move by action of cilia Trophozoite and cyst stages Asexual reproduction
Phylum: Apicomplexa (sporozoa)	Single celled Usually inhabit tissue and blood cells Insects and other mammals are involved as part of life cycle May have both sexual and asexual life cycles
Phylum: Microsporidia	
	True eucaryotes with membrane bound nucleus Bacterium-like ribosomes No discernible mitochondria Belong to the kingdom of fungi
Phylum: Platyhelminthes (flatworms)	
Class: Trematoda (flukes)	Multicelled and bilaterally symmetric Most are hermaphroditic Egg, miracidium, cercaria, and adult are life cycle stages Fish, snails, and crabs are involved as intermediate hosts in life cycle
Class: Cestoda (tapeworms)	Multicelled, ribbonlike body Hermaphroditic Egg, larva, and adult worm are life cycle stages Mammals and insects are involved as intermediate hosts in life cycle
Phylum: Aschelminthes	
Class: Nematoda (roundworms)	Adults of both sexes Egg, larva, and adult worm are life cycle stages May have free-living form or may require intermediate host

organisms discussed here live in the large intestine. All amebae possess a trophozoite and cyst stage. *B. hominis* has additional morphologic stages. The trophozoite is the motile feeding stage that reproduces by binary fission. The cyst is an infective, environmentally resistant stage. Multiplication of nuclei in the cyst stage also serves a reproductive function.

Life cycle

The life cycle of amebae is relatively simple, with direct fecal-oral transmission in food or water via the cyst stage and no **intermediate hosts**. Humans ingest the infective cysts, which excyst in the intestinal tract, and the emerged trophozoites multiply by binary fission. Trophozoites colonize the cecal area. Fig. 28.2 illustrates a generalized life cycle for amebae and the extraintestinal phase of *E. histolytica*.

Treatment

Treatment is administered only for *E. histolytica* infections; treatment for nonpathogens is not indicated. Luminal amebicides, such as paromomycin, are given to carriers in nonendemic areas to prevent the invasive phase and reduce the risk of transmission. In endemic areas with a high risk of reinfection, treatment may not be indicated. Patients with invasive amebiasis are treated with systemic drugs, such as metronidazole and luminal amebicides. In cases of *Blastocystis* spp. infection, treatment may be indicated if the patient is symptomatic.

Entamoeba histolytica and related organisms

E. histolytica and *E. dispar* are morphologically identical organisms that differ in their effects on the host. *E. histolytica* is a recognized pathogen, while *E. dispar* is nonpathogenic. Recently, *E. moshkovskii* and *E. bangladeshi*, which are also morphologically identical to *E. histolytica*, have been identified as belonging to this complex. It is unclear if the latter two organisms are pathogenic; however, some studies indicate *E. bangladeshi* is an agent of diarrhea.

Historically, studies demonstrated that many people were infected with an organism identified as *E. histolytica*. However, only about 10% of these individuals developed clinical symptoms or invasive disease. It was thought that perhaps two strains of the organism existed, one pathogenic and one nonpathogenic. For many years, this hypothesis remained unproven because there was no way to differentiate the strains morphologically.

Clinical studies that took place after the emergence of human immunodeficiency virus (HIV) provided the impetus to explain the discrepancy. Studies using electrophoresis identified differing isoenzyme (zymodeme) patterns between organisms that caused clinical symptoms and those found in asymptomatic persons. The studies showed that most strains in asymptomatic cyst passers and in most men who have sex with men were nonpathogenic, whereas several strains from areas with high rates of endemic disease were pathogenic. Further immunologic and DNA probe studies supplied additional evidence, such as differences in epitopes of the galactose-binding lectin, differences in surface antigens, and differences in gene expression. On the basis of this evidence, the noninvasive organism, formerly referred to as *nonpathogenic E. histolytica*, was named *E. dispar*. The pathogenic organism continued to be referred to as *E. histolytica*. *E. moshkovskii* is morphologically identical to *E. histolytica* and *E. dispar* and

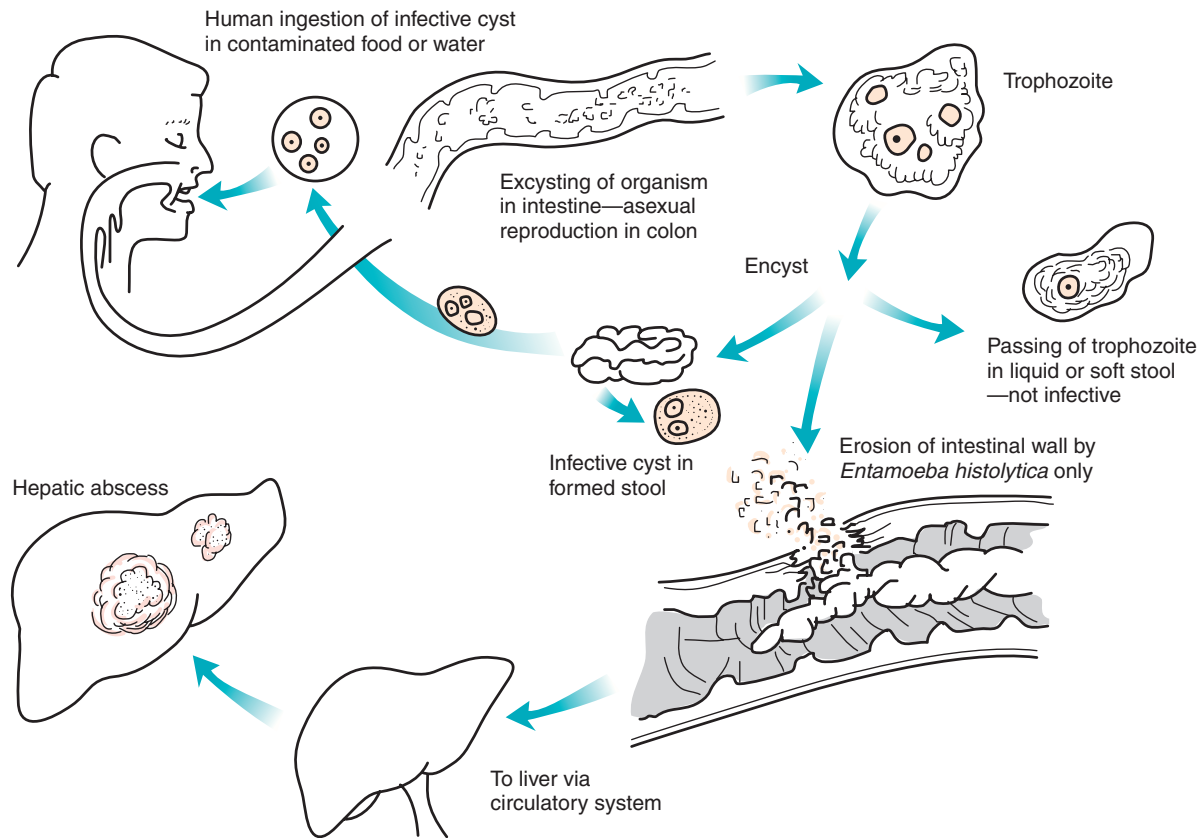


Fig. 28.2 Generalized life cycle of intestinal amoeba.

was thought to be a free-living organism. Subsequent studies identified it as a GI organism reported from humans in many countries. Its true significance is unknown, but initial evidence suggests that it is nonpathogenic.

The organisms are morphologically identical and must be distinguished on the basis of identification of surface antigens. However, the presence of ingested erythrocytes in the trophozoite stage will distinguish *E. histolytica* from the other species. *E. histolytica* is found worldwide, but especially in the tropics and subtropics. It is a major protozoan pathogen for humans, causing an estimated 500 million infections and 50 million cases of colitis and hepatic abscesses annually. It ranks third behind malaria and schistosomiasis as a cause of death resulting from a parasitic infection, accounting for an estimated 40,000 to 100,000 annual deaths. Due to misdiagnosis and under-reporting, the prevalence is thought to be much greater. Prevalence of infection differs according to socioeconomic levels and sanitary practices; infection is more common in developing countries. Travelers to those areas are at increased risk of acquiring infection. Other factors that may influence susceptibility include specific human leukocyte antigen (HLA) phenotypes and mutations in the hormone leptin. Although the primary mode of transmission is fecal-oral transmission, the organism has also been identified as a sexually transmitted agent among men who have sex with men. HIV infection does not appear to increase the risk of invasive disease.

Clinical infection

E. histolytica normally subsists on intestinal bacteria and partially digested food of the host. The pathogenicity of

E. histolytica is reflected, however, in its ability to cause invasive intestinal amebiasis and extraintestinal amebic infections. The organism adheres to the mucous layer and cells of the intestine using surface lectins with affinity for galactose and *N*-acetyl-galactosamine. It invades and disrupts the mucosal barrier, produces contact-dependent killing, and induces apoptosis of the intestinal cells. A protein known as *amoebapore* will create a channel in the cell that allows rapid influx of calcium, resulting in cell death. The organism lyses and phagocytizes the cell. Invasion of the deeper layers of the intestinal wall is mediated by cysteine proteases that destroy collagen and fibronectin. Evidence suggests that trophozoites also use trogocytosis (the process of ingesting pieces of living cells) to disrupt cell membranes.

The host secretes proinflammatory cytokines, leading to an acute inflammatory response and migration of neutrophils and macrophages into the tissue. The organism can also secrete chemoattractants for neutrophils. It kills these cells by contact-dependent lysis, and the subsequent release of lysozymes, superoxides, and collagenases from the neutrophil granules produces additional damage to the intestinal mucosa.

The host's innate and acquired immune responses come into play to prevent colonization. Mucin in the intestinal mucus competes for attachment to the lectin and protects epithelial cells from attack. The host may have intestinal immunoglobulin A (IgA) that helps prevent colonization and repeat infection, and antigen-specific T cells secrete cytokines that have direct cytotoxicity for the trophozoites. The trophozoite is, however, resistant to complement-mediated lysis.

E. histolytica infection presents in several ways: asymptomatic colonization, amebic dysentery (colitis), and extraintestinal amebiasis. In almost 90% of infections, the patients are asymptomatic. Individuals who are colonized but remain asymptomatic pose a great risk to others because they are cyst passers and therefore infective. In symptomatic individuals, clinical infection may appear in an acute or chronic form. Those with increased risk for severe disease include young individuals, older adults, malnourished individuals, and those receiving immunosuppression therapy. In acute infections, the patient may experience vague abdominal symptoms, such as tenderness and cramping, fever, and up to 20 diarrheic stools per day that contain trophozoites, blood, and mucus. Some stools contain Charcot-Leyden crystals, which are breakdown remnants of eosinophils; their presence in the stool is suggestive of an intestinal parasitic infection but is not specific for *E. histolytica*.

In severe *E. histolytica* infection, the patient may shed pieces of intestinal mucosa. Some patients develop an *ameboma* (amebic granuloma), a tumorlike lesion that forms in the submucosa of the intestine. This represents an area of chronic lysis and infiltration with neutrophils, lymphocytes, and eosinophils. In chronic infections, however, there may be alternating asymptomatic periods and diarrheal episodes.

The characteristic lesion in the intestinal mucosa, a flask-shaped ulcer, is a result of lysis of the intestinal mucosa. The lesion shows a pinpoint ulceration on the mucosal surface and a gradual widening in the submucosal areas and lamina propria as the parasites invade the tissue. The organisms may completely erode the intestinal mucosa and enter the circulation. To survive and colonize the liver, they must resist both antibody and complement activity. Cysteine proteases can inactivate IgG. The trophozoite also uses surface receptor capping—a process of removing surface receptors that have been recognized by antibody. These receptors move to the posterior of the organism and are shed in vesicles. When the trophozoite avoids destruction in the circulation, extraintestinal amebiasis often results. The organ usually colonized is the right lobe of the liver because organisms enter the portal circulation and are then trapped in the venules of the liver. Patients with hepatic abscesses may have symptoms, such as fever, chills, and pain in the upper right quadrant, or they can be asymptomatic. Weight loss, increased WBC counts, or

elevated liver enzyme levels may be present. Jaundice is usually absent. Lung abscesses may be seen as the result of penetration of the diaphragm by amebae from hepatic abscesses or from hematogenous spread. Invasion of the lung can cause the patient to have chest pain, dyspnea, and a productive cough.

Laboratory diagnosis

Patients with diarrhea are most likely to have trophozoites in the stool that may be seen in direct wet mounts or trichrome-stained smears. Sigmoid biopsies may be used to demonstrate the characteristic morphology of the intestinal ulcers or to identify trophozoites in tissue when none can be isolated from the stool specimen. [Table 28.3](#) summarizes the characteristics of *E. histolytica* trophozoites and cysts and compares *E. histolytica* with other amebae. In a direct saline wet mount of a diarrheic stool, the trophozoite of *E. histolytica* may exhibit a progressive, directional motility by extending long, thin pseudopods. The size of the organism ranges from 10 to 50 μm , averaging 15 to 25 μm . The organism is refractile, with the characteristic “bull’s eye” nucleus, consisting of a small central karyosome. Even, fine peripheral chromatin may be only slightly visible.

In a trichrome-stained smear, the cytoplasm of the trophozoite appears clean and free from ingested bacteria and vacuoles. Finely granular nuclear chromatin is evenly distributed on the nuclear membrane, and the small central karyosome stains dark purple-red. Ingested RBCs are diagnostic for *E. histolytica* trophozoites but usually are not seen. If there are no ingested RBCs, the organism must be reported as *E. histolytica*/*E. dispar*. The World Health Organization (WHO) and the Pan American Health Organization recommend that *E. moshkovskii* also be included. Immunoassays are necessary to differentiate these species. [Fig. 28.3](#) shows trichrome-stained trophozoites of *E. histolytica*. The trophozoite in [Fig. 28.3B](#) contains an ingested RBC.

Cysts of *E. histolytica* may have one to four nuclei, each with a small central karyosome and fine, evenly distributed peripheral chromatin. The cytoplasm may occasionally contain chromatoidal bars composed of ribonucleic acid. The average size of the cyst is 10 to 20 μm ([Fig. 28.4](#)). The chromatoidal bars are cigar shaped, with rounded ends. Young cysts often contain multiple bars, and mature cysts may show only one.

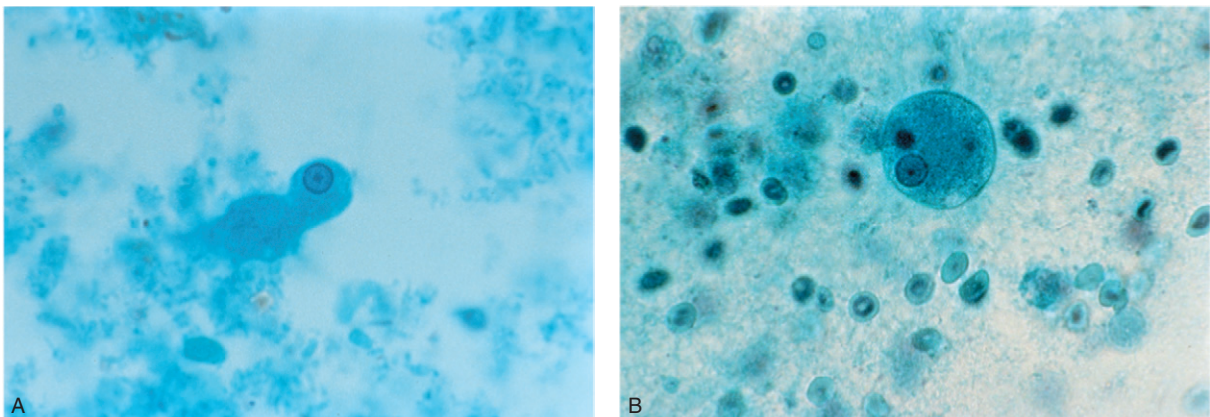
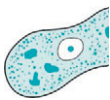
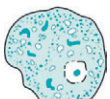


Fig. 28.3 A, *Entamoeba histolytica* trophozoite (trichrome stain). B, *E. histolytica* trophozoite. Notice the darkly staining, ingested red blood cell near the nucleus (trichrome stain). (A, $\times 1000$; B, $\times 1000$.)

Table 28.3 Comparisons of amebae

Organism	Trophozoite			Trophozoite and cyst nuclear structure	Cyst			
	Size (μm)	Motility	Cytoplasm		Size (μm) and shape	No. of nuclei in mature cyst	Chromatoidal bars	Glycogen vacuole
<i>Entamoeba histolytica</i> 	15–25	Progressive, directional	Finely granular May contain ingested red blood cells	Small, central karyosome Fine, evenly distributed peripheral chromatin	10–20, round	Four 	Rounded Elongated Usually seen	Not usually seen Diffuse in young cyst
<i>Entamoeba coli</i> 	15–50	Nondirectional	Vacuolated Ingested bacteria	Large, eccentric karyosomes Coarse, uneven peripheral chromatin	15–25, round	Eight 	Elongated Splintered ends Not always seen	Not seen
<i>Entamoeba hartmanni</i> 	4–12	Nondirectional	Finely granular	Small, central karyosomes Fine, evenly distributed peripheral chromatin	5–10, round	Four 	Rounded ends Elongated Not always present	Not seen
<i>Endolimax nana</i> 	5–12	Nondirectional	Vacuolated May contain ingested bacteria	Large, irregularly shaped karyosome No peripheral chromatin	5–12, oval	Four 	Not present	Not seen
<i>Iodamoeba bütschlii</i> 	6–20	Nondirectional	Vacuolated May contain ingested bacteria	Large karyosome surrounded by achromatic granules No peripheral chromatin	6–15, oval or irregular	One 	Not present	Single, defined

In an iodine wet mount, the nuclei appear as yellowish refractile bodies within the cyst; chromatoidal bars do not take up stain and appear as colorless areas. With trichrome stain, the cyst stains light green to gray; nuclear material and chromatoidal bars stain dark purple-red. Young cysts may show discrete glycogen masses that stain light brown in an iodine wet mount, but in the more mature cyst, the glycogen is diffuse. Cysts can persist for 2 to 4 weeks in a moist environment but may be killed by drying, temperatures over 55° C, superchlorination, or addition of iodine to drinking water.

Amebic ulcers of the liver often are detected with ultrasonography or radiography. Subsequent aspiration of the abscess may yield motile trophozoites and necrotic material composed of lysed cells. Serologic methods of detecting antibody to *E. histolytica* are available; the results are positive in more than 90% of patients with extraintestinal disease. The antibody levels increase after tissue invasion but are not protective. Tests for antibodies, however, are not particularly useful in distinguishing between past and current infection because antibodies can persist for years after an infection has resolved. In addition, these tests provide limited information in patients from endemic areas.

Tests that detect *E. histolytica* antigen in stool provide evidence of current infection. Some kits require a large number of organisms to be present for a positive result; however, antigen detection is still considered more sensitive than a microscopic examination of feces. These tests use the EIA method with

monoclonal antibodies to proteins (e.g., serine-rich antigen) or the galactose/*N*-acetylgalactose adhesion lectins. A number of kits detect *E. histolytica* antigens without excluding *E. dispar*; others differentiate *E. histolytica* from *E. dispar*. Point-of-care tests using immunochromatographic techniques assist with the rapid diagnosis of *E. histolytica* infection. Antigen detection assays can be used on other specimens besides feces. For example, testing serum can be effective in diagnosing amebic liver abscess. Multiplex molecular assays for GI syndromic testing are also available, and *E. histolytica* is often included.

Entamoeba hartmanni

E. hartmanni, once known as *small race E. histolytica*, is a nonpathogen. It generally resembles *E. histolytica* in a trichrome-stained smear but is more likely to have an eccentric karyosome or uneven peripheral chromatin resembling that of *Entamoeba coli*. Size is a major determinant in differentiating *E. histolytica* from *E. hartmanni*. The average size of the trophozoites of *E. hartmanni* is 4 to 12 μm ; trophozoites with an average size larger than 12 μm are identified as *E. histolytica* or *E. dispar*. *E. hartmanni* cysts measure 5 to 10 μm ; those of 10 μm or larger are identified as *E. histolytica*, *E. dispar*, or *E. moshkovskii*. Fig. 28.5A shows the trichrome-stained trophozoite, and Fig. 28.5B shows an enlarged view of *E. hartmanni* demonstrating the irregular peripheral chromatin. Fig. 28.6 shows the cyst of *E. hartmanni*, with three nuclei visible and at least one chromatoidal bar.

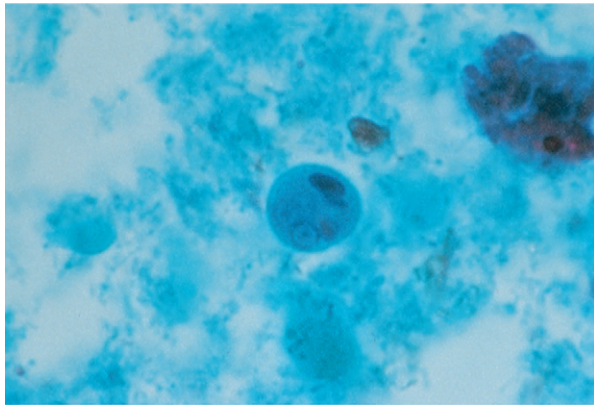


Fig. 28.4 *Entamoeba histolytica* cyst with round-end chromatoidal bars. Two nuclei are visible (trichrome stain, $\times 1000$).

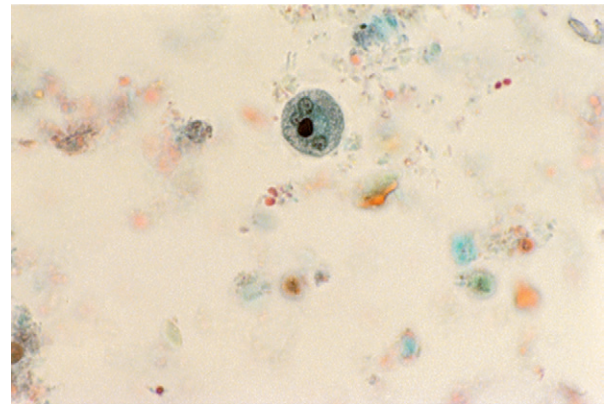


Fig. 28.6 *Entamoeba hartmanni* cyst (trichrome stain, $\times 1000$).

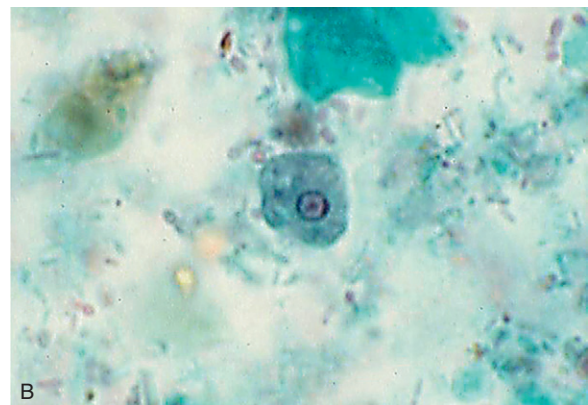
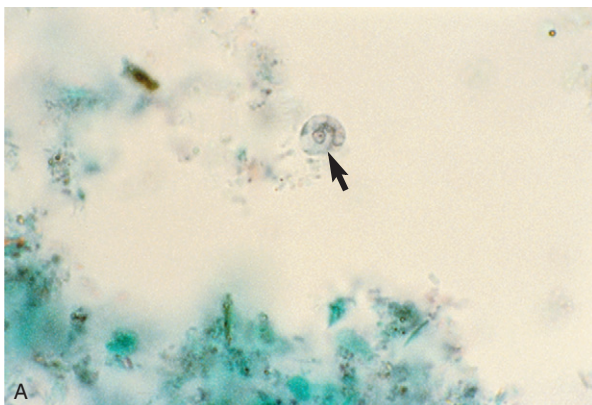


Fig. 28.5 A, *Entamoeba hartmanni*. Identified by the arrow (trichrome stain). B, Enlarged view of *E. hartmanni* trophozoite. (A, $\times 400$; B, $\times 1000$.)

Entamoeba coli

Entamoeba coli is a commonly found intestinal commensal transmitted by the ingestion of cysts in fecally contaminated food or water. The average size of the trophozoite is 15 to 50 μm , with most measuring 25 μm (Fig. 28.7A). The nuclear structure is characterized by a large eccentric karyosome and coarse, uneven peripheral chromatin on the nuclear membrane. Fig. 28.7B shows an enlargement of the organism demonstrating the characteristic nuclear morphology. The motility of the trophozoite in a wet preparation is sluggish and nondirectional. In a permanently stained preparation, the cytoplasm of the trophozoite may stain a dark purplish gray and contains vacuoles and ingested materials.

The mature cyst has eight nuclei; the immature cyst may have one or two large nuclei, with a large glycogen vacuole. Cysts often stain darkly and unevenly, giving an irregular appearance to the cyst. The large eccentric karyosome is visible, but peripheral chromatin surrounding the entire nucleus may be difficult to see. Chromatoidal bars, when present, have a pointed, splintered appearance. The average size of the cyst is 15 to 25 μm . Fig. 28.8A shows a merthiolate-iodine-formalin wet mount of an *E. coli* cyst, and Fig. 28.8B shows a trichrome-stained cyst.

Endolimax nana

The trophozoite of *E. nana* has a large karyosome, with no peripheral chromatin on the nuclear membrane. The trophozoite ranges in size from 5 to 12 μm , with the average being less

than 10 μm . Fig. 28.9 shows three trophozoites. The cytoplasm is granular and vacuolated. In a wet preparation, the motility is sluggish. In a wet mount, it may be difficult to distinguish the large karyosome of *E. nana* from the karyosome of *E. hartmanni*, and the organisms may be misidentified. The cyst of *E. nana* is oval or spherical, 5 to 12 μm , and has up to four large nuclei (Fig. 28.10). Fig. 28.10B shows an enlargement of the cyst, with three of the characteristic large buttonhole nuclei easily visible. A fourth is partially visible. At least one of the nuclei shows evidence of the thin nuclear membrane.

Iodamoeba bütschlii

Iodamoeba bütschlii is less commonly encountered compared with other nonpathogenic amebae. The nucleus is composed of a single, irregularly shaped karyosome surrounded by achromatic granules and a thin nuclear membrane, with no peripheral chromatin. The trophozoites of *I. bütschlii*, which are 6 to 20 μm in size, show a vacuolated cytoplasm in a permanently stained smear (Fig. 28.11). The oval cyst is 6 to 15 μm (average, 9 to 10 μm) in size and contains a single large karyosome and a large, well-defined glycogen vacuole. The vacuole stains dark brown in an iodine wet mount and appears empty in a permanently stained smear. Fig. 28.12 demonstrates an iron hematoxylin-stained cyst of *I. bütschlii*.

Blastocystis spp.

The taxonomy of *Blastocystis* is unresolved. While originally classified as a yeast, the genus *Blastocystis* has recently

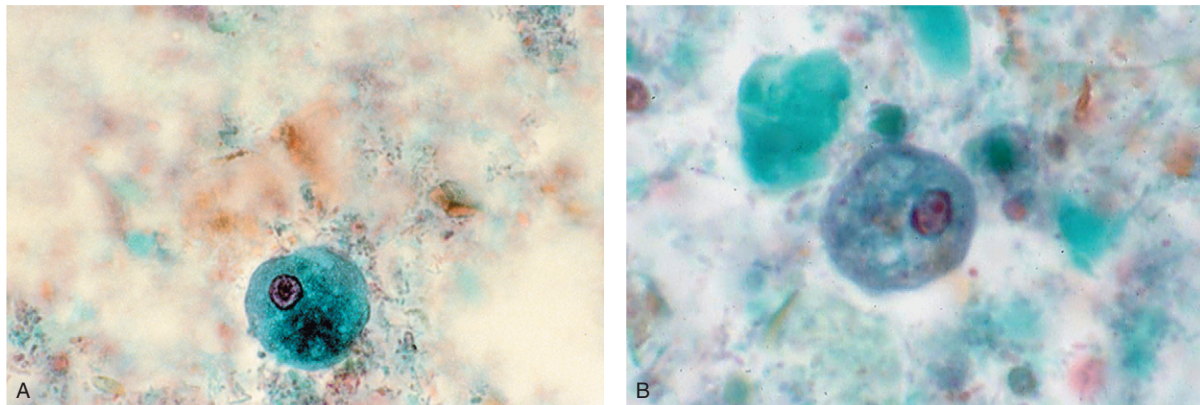


Fig. 28.7 A, *Entamoeba coli* trophozoite. Notice the darkly staining, highly vacuolated cytoplasm (trichrome stain). B, Enlarged view of *Entamoeba coli* trophozoite to demonstrate peripheral chromatin (trichrome stain). (A, $\times 1000$; B, original magnification $\times 1000$.)

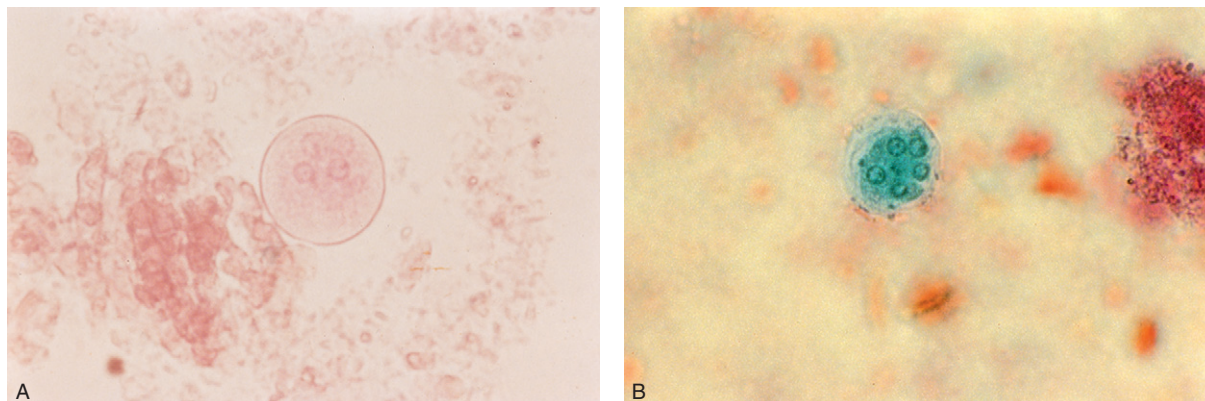


Fig. 28.8 A, *Entamoeba coli* cyst (merthiolate-iodine-formalin) wet mount. B, *Entamoeba coli* cyst with five nuclei visible (trichrome stain). (A, $\times 1000$; B, $\times 400$.)

undergone taxonomic reclassification. Genetically, *Blastocystis* spp. closely resemble *Proteromonas lacerate*, a flagellate. However, *Blastocystis* is nonmotile and does not have flagella. Ribosomal ribonucleic acid (rRNA) analysis indicates that it is related to the stramenopiles (brown algae and water molds). Studies indicate that at least 12 different species are found in humans and other animals, and *Blastocystis hominis*

is no longer considered a valid species name. Clinical microbiologists should simply report isolates as *Blastocystis* sp.

Blastocystis is one of the most common intestinal protozoa and has a prevalence rate of greater than 50% in developing countries. Infection occurs in immunocompetent and immunocompromised individuals. *Blastocystis* has come to prominence as a possible cause of diarrhea in humans, although controversy concerning its pathogenicity persists because of conflicting results from numerous studies. Although not considered a common cause of diarrheal disease, this organism,

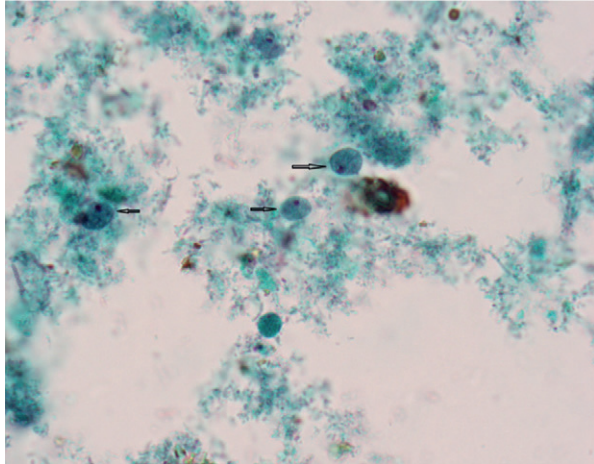


Fig. 28.9 *Endolimax nana* trophozoites, identified by arrows (trichrome stain, ×1000).

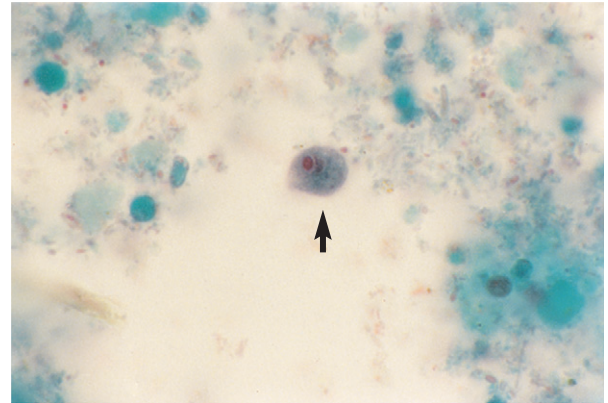


Fig. 28.11 *Iodamoeba bütschlii* trophozoite, identified by the arrow (trichrome stain, ×1000).

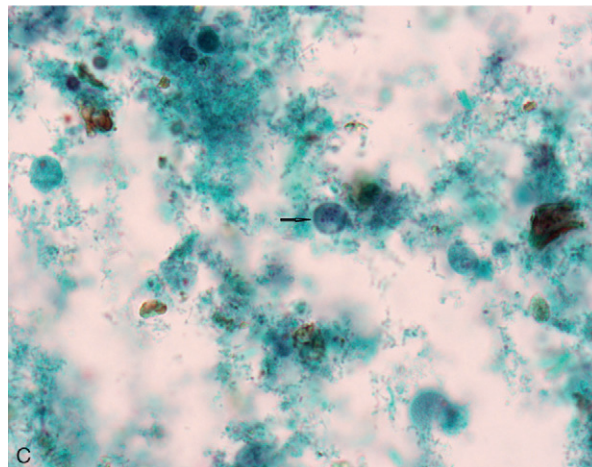
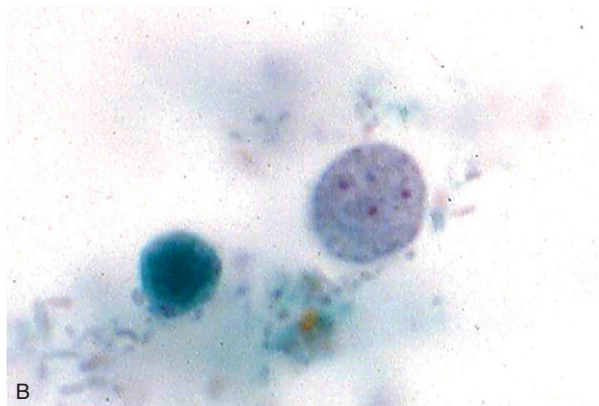
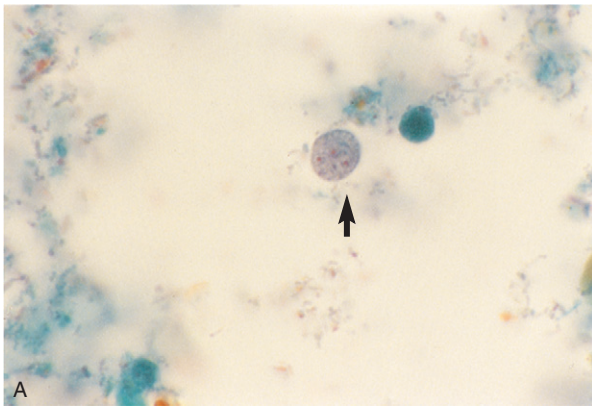


Fig. 28.10 **A**, *Endolimax nana* cyst, identified by the arrow (trichrome stain). **B**, Enlarged view of *E. nana* cyst (trichrome stain). **C**, Cysts (identified by arrow). (**A**, ×1000; **B**, original magnification, ×1000, **C**, original magnification ×1000.)

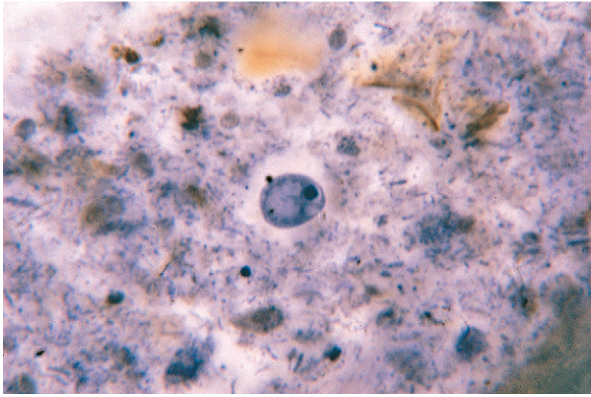


Fig. 28.12 *Iodamoeba bütschlii* cyst with prominent glycogen vacuole (iron hematoxylin stain, $\times 1000$).

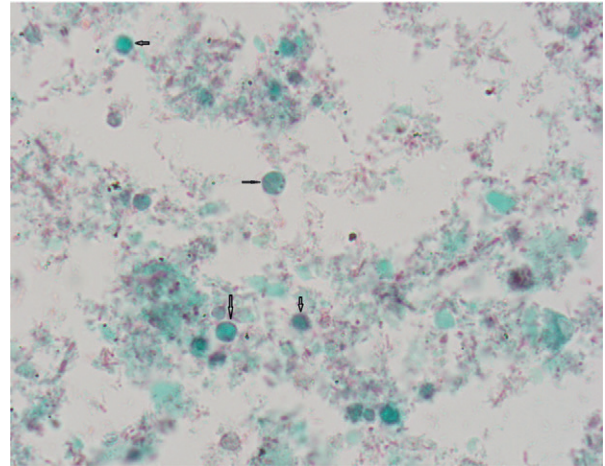


Fig. 28.13 *Blastocystis* sp. vacuolar form, identified by arrows (trichrome stain, $\times 1000$).

nevertheless, has been found in patients who have abdominal pain and diarrhea but no other intestinal pathogens. The organism has been associated with chronic infection in some patients. Infected individuals often have a history of travel abroad and, most frequently, of consuming untreated water. Other patients have a history that involves handling of animals. Some studies linked the presence of *B. hominis* to colitis and irritable bowel syndrome (IBS), whereas others have not. Others have suggested that *B. hominis* has no role as a pathogen.

Several subtypes have been identified based on ribosomal DNA (rDNA), which could explain the differences in clinical presentation. The STI-4 strain is seen primarily in humans, whereas other strains are seen in a variety of animals. In addition, the presence of specific cysteine proteases that may function as virulence factors could affect pathogenicity. These proteases are essential for host invasion and trigger proinflammatory cytokines, such as interleukin (IL)-8. Some studies have suggested that certain genotypes may be part of the intestinal microbiota and contribute to intestinal homeostasis. Patients infected with *Blastocystis* may present with diarrhea, abdominal pain, bloating, anorexia, and flatulence. Laboratories often report the presence of the organism quantitatively, with the presence of more than five organisms per high-power field and no other known enteric pathogens in symptomatic patients considered evidence of *Blastocystis* as the cause of GI symptoms.

Blastocystis sp. is a polymorphic organism that exists in four forms: ameboid, granular, central vacuolar (central body form), and cystic. The central body form is the most commonly identified. The life cycle is not completely known, and revisions continue to occur. The cyst is ingested and is infective. The organism excysts in the large intestine, where it transforms into the central vacuolar form. It is not known what triggers the change into other forms. It is known that the organism encysts as stool passes through the large intestine.

The average size for the vacuolar form is 5 to 15 μm , but up to 20% of organisms are smaller than 5 μm , and a few approach 100 μm . The organism is round and has a ring of cytoplasm lining the inside of the plasma membrane, with as many as four nuclei present, which are usually pushed to the side. There is a large central vacuole that occupies up to 90% of the cell volume and may play a role as a storage organelle (Fig. 28.13). The granular form is similar but possesses

granules in the central vacuole and cytoplasm. The ameboid form, which may be seen in diarrheic stools, is 3 to 8 μm and often lacks a vacuole but possesses several pseudopods. In an iodine mount, the cytoplasm and central area of the vacuolar form stain brown. With the trichrome stain, the cytoplasm stains dark green and the central area may stain pale to intensely green, with the nuclei staining dark purple to black. The cyst form can be found in stool and in culture. The cyst stage is small (3 to 5 μm), is oval to round, and possesses one to four nuclei and multiple vacuoles. Because of its small size, it may resemble fecal debris. It is resistant to the usual chlorine levels in drinking water, and water has been implicated in transmission of the organism.

Tissue amebae

Free-living, thermotolerant amebae can tolerate a wide range of temperatures, pH, and salinity. They are found in soil and water (e.g., freshwater sources, domestic water supplies, sewage, swimming pools), usually feeding on bacteria in the environment, but are known to cause human disease. They can gain access to the CNS by inhalation into the upper respiratory tract followed by penetration of the nasal mucosa, which allows them to travel along the olfactory nerve to the brain, or by hematogenous spread from the lungs or skin lesions. Although the number of infections caused by these organisms is low compared with those caused by intestinal protozoans, they are very difficult to diagnose and treat and are associated with a high mortality rate. *Naegleria fowleri* and *Acanthamoeba* spp. have been recognized for many years as the amebae most commonly associated with CNS invasion in humans. The media sometimes refer to *N. fowleri* as the "brain-eating ameba."

The ameboflagellate *N. fowleri* is the only one of about 30 species of *Naegleria* that has been identified as a human pathogen. It is the causative agent of primary amebic meningoencephalitis (PAM), a rapidly fatal condition involving the CNS. *Acanthamoeba* spp. have been associated with a more chronic condition, granulomatous amebic encephalitis (GAE), especially in individuals with impaired cell-mediated immunity. In addition, *Acanthamoeba* spp. have been linked with amebic keratitis in soft contact lens wearers and with

Table 28.4 Comparison of central nervous system infections caused by amebae

Parameter	Primary amebic meningoencephalitis	Granulomatous amebic encephalitis
Causative agent	<i>Naegleria fowleri</i>	<i>Acanthamoeba</i> spp. and <i>Balamuthia mandrillaris</i> ^a
Stages in:		
Cerebrospinal fluid	Trophozoite	Trophozoite
Brain biopsy	Trophozoite	Trophozoite and cyst
Characteristics	Trophozoite, 10–35 μ m Large karyosome Broad pseudopods	Trophozoite, 15–45 μ m Spinelike pseudopod Cyst, 15–20 μ m Wrinkled double wall
Entry	Nasal passage—olfactory nerve to CNS	Lungs and skin with hematogenous spread to CNS
Clinical course	Fulminant (death within 1 week of onset)	Slow and chronic
Population at risk	Children to young adults, healthy (history of water activities in stagnant, warm water)	Immunocompromised
CNS, Central nervous system.		
^a The pathogenesis and morphology of <i>Acanthamoeba</i> spp. and <i>Balamuthia mandrillaris</i> are similar. In some cases, <i>Balamuthia</i> appears to have more than one nucleolus in tissue sections compared to one found in <i>Acanthamoeba</i> .		

cutaneous infections in patients with acquired immunodeficiency syndrome (AIDS). A comparison of the CNS infections caused by *N. fowleri* and *Acanthamoeba* spp. is provided in Table 28.4. *Balamuthia mandrillaris*, another free-living ameba, is primarily associated with GAE and cutaneous lesions in humans. In addition, *Sappinia* spp. and *Paravahlkampfia francinae*, also free-living amebae, have been identified in rare cases of encephalitis and meningitis.

Naegleria fowleri

Although PAM has been reported from many countries worldwide, it is a relatively rare infection. However, many studies have shown the presence of antibodies directed against *N. fowleri* in healthy individuals. In the United States, between 1962 and 2021, there were 154 reported cases, with only four survivors. PAM is most frequently reported in Texas (40 cases), Florida (36 cases), and California (10 cases) but has been reported as far north as Minnesota. It occurs in healthy, immunocompetent children and young adults with no predisposing condition. A common factor in infection is the report of recent swimming or other water-related activities in warm, artificial lakes or brackish or muddy water or exposure to bottom sediment. Waterskiing, wakeboarding, or other activities that increase the chances of forceful entry of water into the nose may facilitate infection. It is not known why only a few individuals are infected in spite of such

common exposure. In recent years, there have been several cases related to sinus irrigation using tap water.

The life cycle of *N. fowleri* is relatively simple, consisting of three stages: (1) free-living amebic trophozoite, (2) transient flagellate form that appears when there is a scarcity of nutrients, and (3) environmentally resistant cyst. The trophozoite enters the nasal cavity through inhalation of contaminated water or soil. The amebic form colonizes the nasal cavity, invades the nasal mucosa, attaches to olfactory nerves, penetrates the cribriform plate, moves along the olfactory nerve to the olfactory bulb, and moves into the arachnoid space. From there, it is free to spread throughout the CNS. Infections are thought to be related to inhalation of a large number of organisms or forceful entry of water into the nose (microtrauma) and to the virulence of the strain.

Clinical infection

Clinically, the disease cannot be distinguished from bacterial meningitis. The incubation period is usually 2 to 3 days but may range up to 2 weeks. Initial symptoms include severe bifrontal headache, fever (38° to 41° C), stiff neck, and nausea and vomiting. CSF will show evidence of increased intracranial pressure, increased levels of neutrophils and protein, and decreased glucose levels. In the late stages, there may be increased numbers of RBCs in the CSF. The organism multiplies in brain tissue, and within 2 to 4 days, the patient can experience drowsiness, confusion, and seizures that can progress to coma. The disease is usually fatal within 1 week of the appearance of clinical symptoms. At autopsy, there will be evidence of trophozoites along with a purulent exudate, edema, and hemorrhagic and necrotic areas of infection in the brain.

N. fowleri is capable of direct cell-to-cell damage resulting in destruction of erythrocytes and other cells, including nerve cells. The invasive properties of the organism are related to its ability to secrete cytotoxic enzymes (e.g., phospholipase, sphingomyelinase) and induce generation of proinflammatory cytokines that facilitate tissue destruction. Macrophages and neutrophils are the primary host defense against the organism because the trophozoites are relatively resistant to the actions of host cytokines. Infection with *N. fowleri* has greater than 95% mortality. Because the symptoms resemble those of bacterial meningitis, specific treatment may be delayed, yet the possibility of cure depends on early diagnosis. Aggressive therapy with intravenous and intrathecal administration of amphotericin B has been used. Rifampin, miconazole, or fluconazole and azithromycin have been used in addition to amphotericin B. Survival in several cases has been linked to additional procedures, including use of medically induced hypothermia and the experimental drug miltefosine.

Laboratory diagnosis

Diagnosis can be made by finding motile amebic trophozoites in CSF. The trophozoite ranges in size from 10 to 35 μ m and moves by explosively extending large, broad pseudopods (lobopodia). The nucleus contains a large central karyosome that is surrounded by a halo. Fig. 28.14 shows a trophozoite of *N. fowleri* as it appears in a CSF specimen stained with iron hematoxylin. In PAM, CSF contains many segmented neutrophils and RBCs and demonstrates elevated protein levels and normal to decreased glucose levels, which are characteristic of bacterial meningitis.

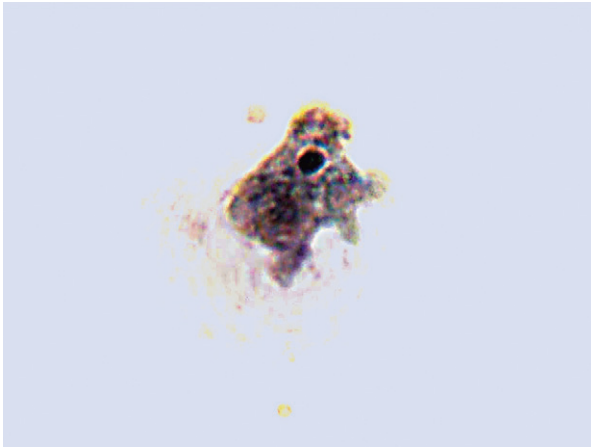


Fig. 28.14 *Naegleria fowleri* trophozoite. Note the prominent karyosome (iron hematoxylin stain, magnification $\times 1000$).

N. fowleri does not stain well with Gram stain, but motile trophozoites can be seen in a wet mount of CSF. They must be carefully distinguished from leukocytes. The trophozoite may also be seen in cytocentrifuged CSF specimens stained with Wright stain. The ameba can be converted to the flagellate stage by adding one drop of CSF sediment to 1 mL of distilled water and incubating it at 37° C. Conversion to the flagellate form occurs in 2 to 20 hours. The organisms can be cultured by overlaying nonnutrient agar with *Escherichia coli* and inoculating the agar with a drop of the CSF sediment and incubating it at 37° C. Clearing of the agar in thin tracks is evidence of the organism feeding on the bacteria. Trophozoites may be observed microscopically. Cysts, which are approximately 10 μm in diameter and have a round, smooth double wall and a single pore, are not seen in clinical specimens. Immunofluorescent staining of CSF with monoclonal antibody can be done in some reference laboratories for detection of the trophozoite. Molecular methods are used to identify strains.

Acanthamoeba

Acanthamoeba spp. have been linked to several clinical conditions, including GAE, cutaneous infections, and amebic keratitis. They have also been identified as the host for several pathogenic bacteria, such as *Legionella* spp., *Vibrio cholerae*, and *Escherichia coli* O157, but the role these may play—if any—in pathogenicity is unknown. Cutaneous acanthamebiasis has been associated primarily with patients with AIDS whose CD4 count is less than 250/ μL . The condition is characterized by chronic nonhealing lesions that may present as nodules, papules, or ulcerations, especially on the extremities and the face. Lesions may develop at the site of inoculation or may occur as a result of hematogenous dissemination from the lungs. Trophozoites and cysts can be seen in biopsy preparations of the lesion. Although it is possible for *Acanthamoeba* spp. to enter the CNS through the nasal passage, the organisms are characterized by hematogenous spread to the CNS from a primary inoculation site in the lungs or skin. The organism penetrates the blood-brain barrier because of changes in the endothelial cell barrier caused by the interaction of the parasitic enzymes and host cytokines.

GAE is subacute; the incubation time is unknown but may range from months to years. Symptoms may include drowsiness, seizures, loss of reflex activity, hemiparesis, headache,

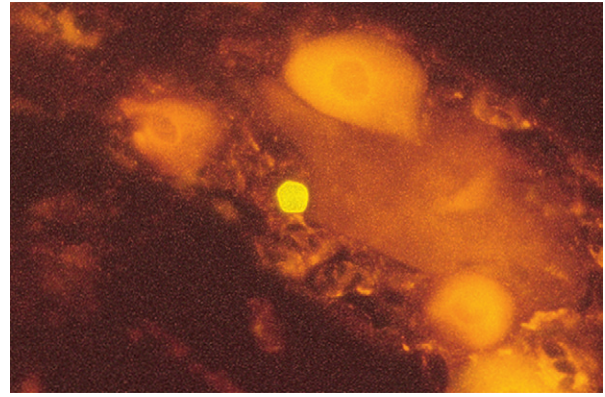


Fig. 28.15 Cornea, scraping, calcofluor white stain, fluorescence microscopy. Polyhedral parasitic cyst (13 μm). Morphology consistent with *Acanthamoeba* cyst.

stiff neck, and personality disorders. The trophozoite is uncommonly seen in CSF, and diagnosis is typically made by brain biopsy demonstrating cysts, trophozoites, or both. Histologic preparations of the brain at autopsy show inflammatory lesions containing many segmented neutrophils, eosinophils, and trophozoites. A specific therapeutic regimen has not been established because most infections have been diagnosed at autopsy. However, disseminated *Acanthamoeba* infections have been treated with amphotericin B, pentamidine isethionate, fluconazole, ketoconazole, and co-trimoxazole (trimethoprim-sulfamethoxazole).

Amebic keratitis, associated with *Acanthamoeba* spp., has been identified in immunocompetent individuals since the 1980s. Individuals who wear contact lenses, especially the soft and extended-wear types, are the primary at-risk group, with greater than 80% of the cases identified. Factors in these infections include improper storage and disinfection procedures, a history of corneal trauma, or wearing contact lenses during swimming, all of which may cause corneal trauma and subsequent colonization by the organism. The organism binds directly to corneal epithelium via acanthopodia and produces proteases and other enzymes that cause cell lysis. Patients experience photophobia, blurred vision, inflammation, ring infiltrates, and pain. Because of the similarity in tissue damage, the infection may initially be confused with bacterial or herpes simplex virus infection, delaying treatment. Phase-contrast microscopy of direct wet mount preparations of corneal scrapings can reveal the trophozoite or cyst. The use of calcofluor white increases detection of the organisms (Fig. 28.15). Permanent stains, such as trichrome and Wright-Giemsa stains, may also demonstrate the trophozoite in clinical specimens (Fig. 28.16).

Isolation of *Acanthamoeba* may be performed in a manner similar to that for *N. fowleri*. *Acanthamoeba* spp. have only two stages: the resistant cyst and the motile trophozoite. The trophozoite ranges from 15 to 45 μm in diameter and has a single nucleus, with a central prominent endosome. Blunt pseudopods and characteristic spinelike projections of the cytoplasm (acanthopodia) may also be seen on a wet mount. The cyst is approximately 15 to 20 μm , spherical, and double walled, with the walls having a wrinkled appearance. Topical applications of chlorhexidine gluconate and ketoconazole have been used to treat cutaneous infections. Corticosteroids are used to reduce inflammation. Despite treatment, patients

with systemic infections have a poor prognosis, and many patients with keratitis lose their sight in the affected eye.

Balamuthia mandrillaris

B. mandrillaris is an emerging opportunistic pathogen that has been identified as a cause of skin lesions and GAE. Unlike *Acanthamoeba*, which has a wide distribution, this organism is found primarily in soil. From 1990 (when it was first linked to human illness) to 2019, this free-living amoeba has caused more than 200 cases worldwide and about 100 cases in the United States. The mortality rate is greater than 95%. In the United States, more than 50% of cases reported occurred in individuals of Hispanic origin. It is unknown if there is a genetic predisposition or if the type of work in which the individuals engage increases exposure risk. Unlike infections with *Acanthamoeba* spp., which occur primarily in immunocompromised hosts, infection with *B. mandrillaris* can occur in immunocompetent and immunocompromised individuals. Humans become infected by inhaling airborne cysts of the organism or by direct inoculation through skin lesions. There have been several reported cases of transmission via organ transplants. No person-to-person spread has been documented.

Skin infections present as a relatively painless nodule. Once the encephalotropic organism has entered the body through the lungs or skin, it spreads hematogenously. Only rarely will it invade the body nasally and spread along nerve fibers to

the olfactory bulb. Once the organism reaches the blood-brain barrier, it is able to bind to microvascular endothelial cells through receptor molecules. The organism produces proteolytic enzymes such as collagenases and metalloproteases that facilitate tissue damage, and the host's inflammatory response helps increase the permeability of the barrier. In the CNS, the organism multiplies by binary fission and causes a necrotizing hemorrhagic infection similar to that caused by *Acanthamoeba* spp. The onset is insidious, with fever, headache, stiff neck, vomiting, and photophobia, and progresses to personality changes and seizures. Onset of symptoms can occur weeks to months after infection, but when the brain is affected, the time to death is short. Treatment involves a multiple antimicrobial regimen, including fluconazole, clarithromycin, and sulfadiazine.

In most cases, infection is identified at autopsy by finding trophozoites and cysts in the tissue. The trophozoite is 30 to 60 μm in diameter, with broad pseudopods and a large single nucleus, with multiple nucleoli. The cyst is round, 10 to 30 μm in diameter, and it has a single nucleus and is multiwalled. The CSF will usually exhibit increased protein levels, normal or decreased glucose levels, and increased levels of lymphocytes. The culture methods using nonnutritive agar seeded with *Escherichia coli* are nonproductive because this organism will not feed on gram-negative bacteria. Immunohistochemical or nucleic acid assays are often needed to identify *B. mandrillaris*.

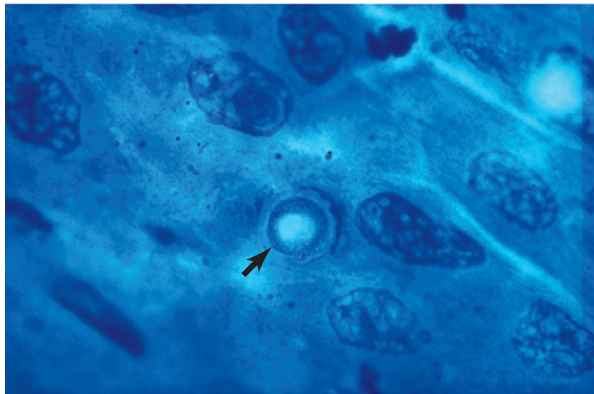


Fig. 28.16 Cornea, scraping, Wright-Giemsa stain, light microscopy Parasitic precyst (13 μm) (arrow). Morphology consistent with *Acanthamoeba* spp.

Ciliates

Only one ciliate, *Neobalantidium coli* (formerly *Balantidium coli*), is considered pathogenic for humans. Pigs are the natural host for this organism, and humans serve as accidental hosts. The organism lives in the large intestine, where it may cause mucosal lesions but not extraintestinal infections. Most people with this infection are asymptomatic, but the organism can cause a self-limiting diarrhea with nausea, vomiting, and abdominal tenderness.

The life cycle is similar to that of the amoebae, with the cyst being the infective stage. This is the largest of the protozoa, and the trophozoite is covered with short cilia. The oval trophozoite (Fig. 28.17A) demonstrates two size ranges: (1) 45 to 60 μm $\times$ 30 to 40 μm and (2) 90 to 120 μm $\times$ 60 to 80 μm .

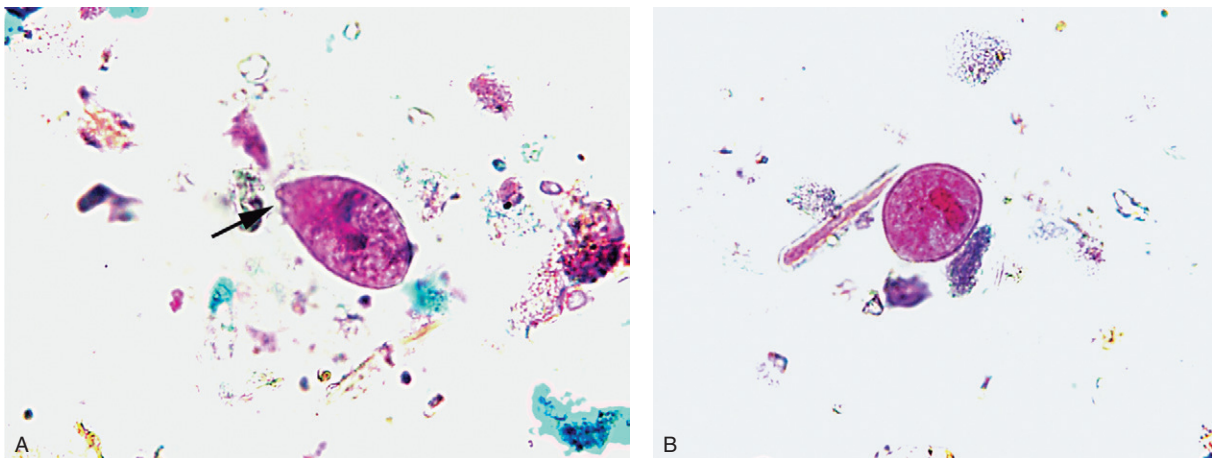


Fig. 28.17 A, *Balantidium coli* trophozoite. The arrow denotes the cytostome (trichrome stain). B, *B. coli* cyst (trichrome stain). (A, $\times 200$; B, $\times 200$.)

The cilia-lined **cytostome** is located at the slightly pointed anterior end. The cytoplasm contains food vacuoles. A small opening at the posterior, the *cytopyge*, is used to expel the contents of food vacuoles. In a wet mount, the cilia are seen propelling the organism with a rotary motion. The rounded, thick-walled cyst (see Fig. 28.17B) averages 45 to 75 μm . Cilia may be seen retracted within the cyst wall. Both stages are characterized by the presence of two nuclei, a kidney bean-shaped macronucleus, and a small, round micronucleus that is usually situated in the small curvature of the macronucleus. The micronucleus is rarely visible in routinely stained smears. Although this is the largest of protozoa, it can be missed during microscopic examination because laboratory scientists are trained to search for protozoan shapes that are typically much smaller.

Pathogenic intestinal and urogenital flagellates

The flagellates constitute another major group of parasites that can inhabit the intestinal tract. The life cycle is relatively simple, resembling that of the amebae (Fig. 28.18). Most flagellates have both cyst and trophozoite stages. *Dientamoeba fragilis*, *T. vaginalis*, and *Trichomonas hominis* lack a cyst stage, however, and the trophozoite of these organisms serves as the infective stage.

Giardia duodenalis, also known as *Giardia intestinalis* or *Giardia lamblia*, originally was considered the only pathogenic intestinal flagellate. However, *D. fragilis* is now recognized as a pathogen. *T. vaginalis* is a pathogen of the genitourinary

tract in men and women. Table 28.5 shows the characteristics of the trophozoite and cyst stages of the intestinal and genitourinary flagellates.

Giardia duodenalis

G. duodenalis has a worldwide distribution and has frequently been identified as the causative agent of outbreaks of gastroenteritis and traveler's diarrhea. It is the most commonly reported intestinal protozoan in the United States. The prevalence rate in underdeveloped countries ranges from 20% to 30%, and the organism is now included in the WHO Neglected Disease Initiative. The main groups at risk for infection are travelers returning from endemic areas, hikers who drink untreated water from streams, and children in daycare centers. Travelers in areas with poor sanitation are often exposed to conditions in which waterborne or foodborne (rare) cysts are ingested. Outbreaks have been reported from swimming pools contaminated with feces. Cysts may remain viable for several months in cold water. They are somewhat resistant to chlorine and iodine, but they are susceptible to desiccation and heating to 50° C.

Some animals, such as beavers, may serve as reservoirs and can be a source of infection for backpackers who drink from streams or rivers. The terms *beaver fever* and *backpacker's diarrhea* have been used to describe the condition in this group of people. Children younger than 5 years of age are at risk for infection, and *G. duodenalis* has caused outbreaks of diarrhea in nurseries and daycare centers as a result of person-to-person contact. Along with *E. histolytica*, *G. duodenalis* has been identified as a sexually transmitted pathogen among those who practice oral-anal sex.

Clinical infection

A low number of cysts, as few as 10, can initiate infection with *G. duodenalis*. Once the cysts are ingested, the gastric acid in the stomach triggers the beginning of excystation. The organism then responds to the slightly alkaline pH in the small intestine and completes the excystation process in the duodenal area, in which the presence of carbohydrates and bile stimulates growth of the trophozoite. Although it does not invade the mucosal surface, it does attach to the surface of columnar epithelial cells. Adherence to the intestinal mucosa is achieved by the ventral sucking disk. Pathologic mechanisms associated with *G. duodenalis* include a mix of human and organism factors. These result in irritation and damage to the mucosa, as well as interference with absorption of nutrients, fats, and fat-soluble vitamins. No toxins or virulence factors have been identified but, there is evidence of inflammatory cell infiltration.

There are eight genetic assemblages—A to H—identified, with subtypes A1 and A2, and B affecting humans. Strain A has a large number of variant surface proteins (VSPs) that can be altered and thus involved in parasite evasion of the host immune response. The intestinal mucous layer; host proteases and lipases; peptides such as defensin and lactoferrin; the presence of secretory IgA; and the influx of mast cells that produce ILs, such as IL-6, help prevent colonization by the organism. The organism increases apoptosis of intestinal cells via caspases, leading to shortening of the intestinal brush border. Damage to the mucosal surface results in atrophy of the microvilli. Bacterial overgrowth may contribute to symptoms.

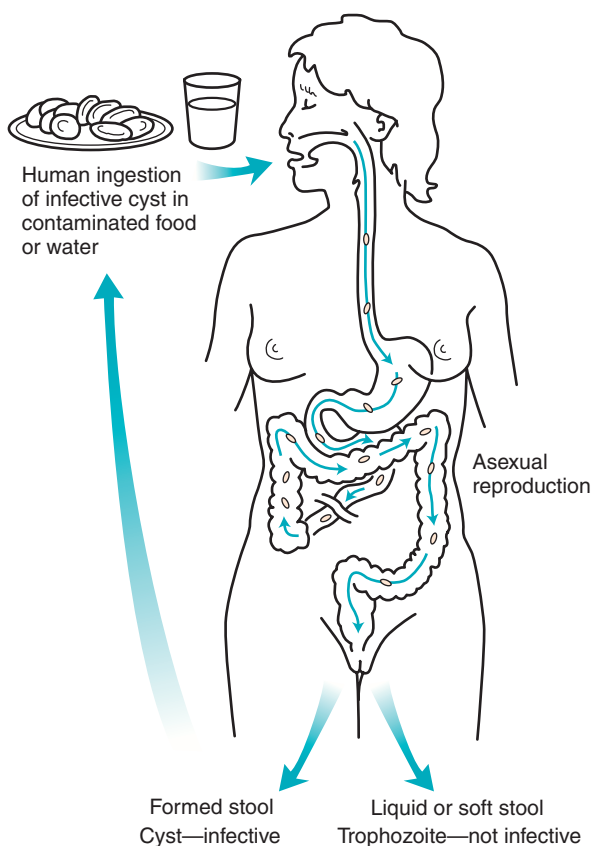
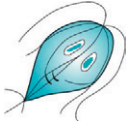


Fig. 28.18 Generalized life cycle for intestinal flagellates.

Table 28.5 Comparison of intestinal and urogenital flagellates

Organism	Trophozoite				Cyst		
	Size (μm)	Motility	No. of nuclei	Other features	Size (μm) and shape	No. of nuclei	Other features
<i>Giardia duodenalis</i> 	9–21 × 5–15	Falling leaf	Two	Sucking disk, ventral surface Parabasal bodies and axonemes	8–12, oval	Four	Cytoplasm retracted from cyst wall Fibrils and flagella inside cyst
<i>Chilomastix mesnili</i> 	10–20 × 3–10	Rotary	One	Spiral groove Cytostome	6–10, lemon-shaped	One	Anterior of cyst has nipple-like protrusion
<i>Trichomonas hominis</i> 	6–14	Jerky	One	Undulating membrane the entire length of the organism Axostyle through body			No cyst stage
<i>Dientamoeba fragilis</i> 	5–12	Nondirectional	Two (20% have one)	Nucleus made of four to eight clustered granules Resembles amoeba			No cyst stage
<i>Trichomonas vaginalis</i> 	7–23 × 5–12; average, 15–18	Jerky, nondirectional	One	Undulating membrane half the length of the body Found in urine			No cyst stage

Clinical presentation ranges from asymptomatic infection to acute infection or chronic infection. In most patients, the acute infection manifests as self-limiting diarrhea, with malaise, cramps, nausea, and abdominal tenderness after an incubation period of 12 to 14 days. Explosive, foul-smelling, nonbloody diarrhea is present, while fever and evidence of inflammation are absent. Symptoms last 1 to 4 weeks, and the patient may lose a significant amount of weight. Patients with secretory IgA deficiency or achlorhydria seem not only to be more apt to acquire the infection but also to develop chronic disease. In chronic giardiasis, there may be a malabsorption-like syndrome, with up to 20% weight loss, fatigue, anorexia, and steatorrhea with large amounts of gas. Fats and vitamin B₁₂ are two of the substances that may be incompletely absorbed in chronic infection, but macrocytic anemia is not common. Children 6 months to 5 years of age are most susceptible to metabolic problems, including iron deficiency, protein malnutrition, and micronutrient deficiency. They may also demonstrate growth and cognitive delay or general failure to thrive. Children with preexisting malnutrition are more likely to develop long-term complications. There have been suggestions that there may also be postinfection complications, such as IBS or reactive arthritis in some individuals. Metronidazole (Flagyl), which has been the drug of choice for treating giardiasis, may be prescribed. Albendazole is another drug that may be used.

Laboratory diagnosis

Feces serve as the usual diagnostic specimen, but shedding of the cysts is irregular, and multiple stool specimens are often required for diagnosis. The trophozoites of *G. duodenalis* are pear shaped or teardrop shaped, have bilateral symmetry, and measure approximately 9 to 21 μm $\times$ 5 to 15 μm . They show a characteristic “falling leaf” motility in a wet mount. In a permanently stained smear, the binucleate organism has been described as having an “old man appearance” (Fig. 28.19A). Two oval nuclei, each with a large central karyosome, are on each side of the midline. Four pairs of flagella, midline axonemes, and two median bodies posterior to the nuclei are also present. A large ventral sucking disk composed of microtubules is used by the organism to attach itself to the intestinal wall. The organism often stains faintly with trichrome stain. Fig. 28.19B shows an enlargement of the trophozoite that reveals several morphologic features. Table 28.5 summarizes the characteristics of *G. duodenalis* and compares them with those of other flagellates. Cysts of *G. duodenalis* are oval and measure approximately 8 to 12 μm $\times$ 7 to 10 μm . There are up to four nuclei, and the cytoplasm is often pulled away from the cyst wall. On a permanently stained smear, the retracted flagella and other internal structures give the cyst a cluttered appearance. Fig. 28.20 show the presence of several cysts of *G. duodenalis*.

EIAs using monoclonal antibodies to detect soluble antigens of *G. duodenalis* in stool are available. Newer lateral flow

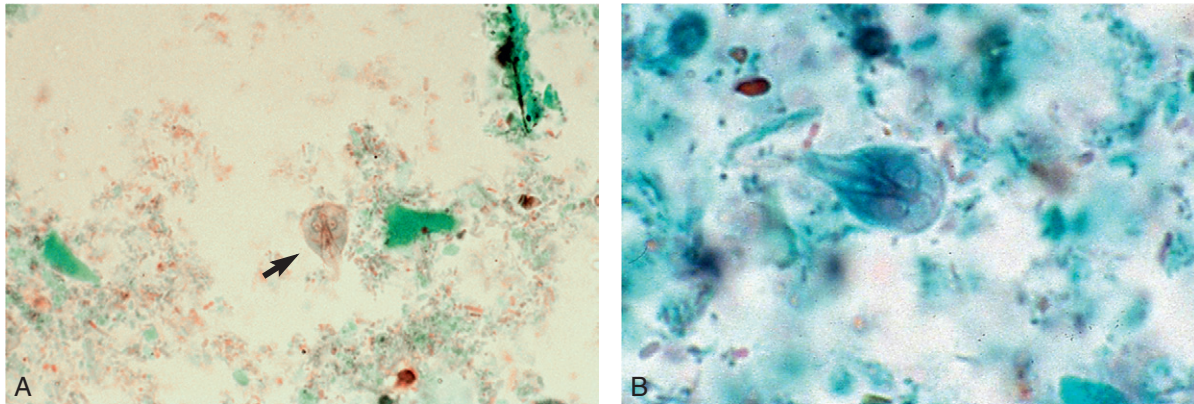


Fig. 28.19 A, *Giardia duodenalis* trophozoite, identified by the arrow (trichrome stain). B, Enlarged view of *G. duodenalis* trophozoite (trichrome stain). (A, $\times 1000$; B, original magnification, $\times 1000$.)

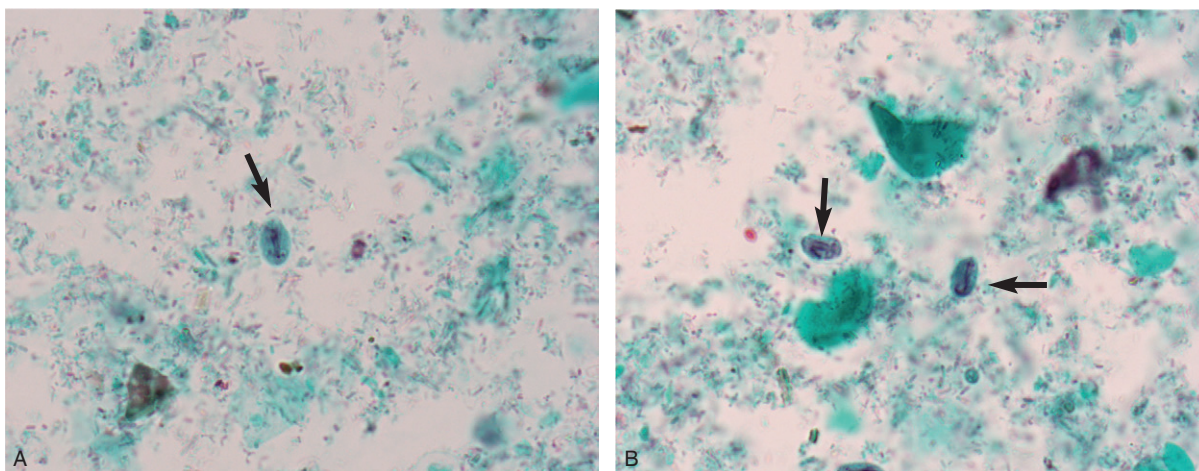


Fig. 28.20 A, *Giardia duodenalis* cysts (arrow). B, *Giardia duodenalis* cysts (arrows). (A, Trichrome stain, $\times 1000$; B, trichrome stain, $\times 1000$.)

immunochromatographic tests that may detect *E. histolytica* and/or *Cryptosporidium* spp. provide results in 15 minutes. DFA tests can be used to identify the cyst of *Giardia* in a stool specimen. Several of these DFA tests also contain a monoclonal antibody that identifies *Cryptosporidium*. The antigen detection assays have a sensitivity of 63% to 100% and a specificity from 90% to 100%. When clinical symptoms persist and the organism cannot be demonstrated in feces or when the patient does not respond to treatment, duodenal aspiration or the Entero-Test has been used to detect the organism.

Dientamoeba fragilis

D. fragilis has a worldwide distribution, with prevalence ranging from less than 1% to greater than 40%. For many years, there were questions about the pathogenicity of *D. fragilis*. The organism was isolated from the stools of patients who were asymptomatic and from those who had GI symptoms, such as abdominal pain or tenderness and diarrhea. Evidence now suggests that the organism has a role as a pathogen in either acute or chronic infections. As with *Blastocystis* infections, links to IBS or irritable bowel disease have been suggested. Case reports show that treatment with metronidazole results in the clearance of the organism and alleviation of symptoms.

Molecular methods show that there are at least two genotypes. While their significance is unknown, genotype 1 is more common. The organism was historically described as lacking a cyst stage, but recent animal studies have identified a cystlike structure in feces. The life span of the trophozoite outside the body is very short, so direct transmission is unlikely. Foodborne or waterborne outbreaks are not common, but some infections have been seen in family groups. Controversy about transmission exists in research studies. Some studies suggest that the trophozoite may be transmitted to humans through ingestion of helminth eggs, especially those of *Enterobius vermicularis*, because co-infection of *D. fragilis* and *E. vermicularis* is more common than co-infection of *D. fragilis* and other organisms. Some, but not all, molecular studies have shown evidence of *D. fragilis* DNA within the pinworm egg. Whether the cystlike structure can be confirmed as the transmission stage remains to be determined.

The morphology of *D. fragilis* closely resembles that of the amebae, hence the name, but electron microscopy studies of ultrastructure, small-subunit rRNA gene analysis, and the lack of a cyst stage all indicate that it is closely related to the trichomonads. *D. fragilis* has apparently permanently lost its flagella. The organism is characteristically binucleate, with 50% to 80% of organisms demonstrating this characteristic. The delicate nuclear membrane has no peripheral chromatin, and the karyosome consists of four to eight discrete granules, one of which is often larger than the others. The size of the trophozoite ranges from 5 to 12 μm , and the cytoplasm contains many food vacuoles and bacteria (Fig. 28.21). This organism degenerates within hours after excretion, and rapid preservation of the stool is important. Since trophozoites do not usually survive the concentration technique, they will only be detected on a permanent stained smear. *D. fragilis* can be difficult to see on a trichrome-stained smear because its outline is often indistinct and blends into the background. No commercial tests are available for *D. fragilis* antigen in stool, so microscopy remains the method of choice.

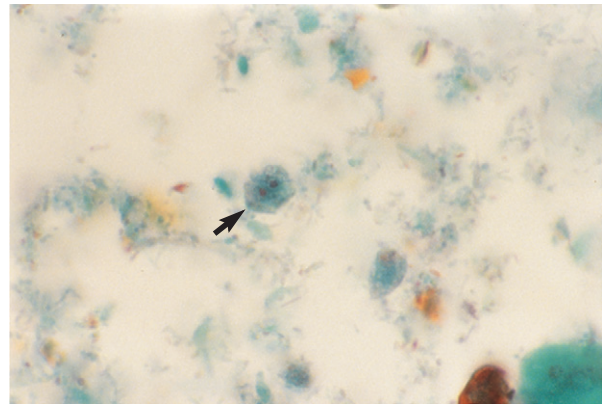


Fig. 28.21 *Dientamoeba fragilis* binucleate trophozoite, identified by the arrow (trichrome stain, $\times 1000$).

Trichomonas vaginalis

T. vaginalis, a pathogen of the urogenital tract in men and women, causes trichomoniasis, one of the most common, nonviral, sexually transmitted diseases (STDs), with an estimate of 180 million cases worldwide. Approximately 3 million to 5 million new cases are reported in the United States each year, with up to two thirds of these occurring in women 15 to 24 years of age. Humans are the only host, and the presence of infection with the organism is associated with other STDs, especially gonorrhea. Trichomoniasis has been increasingly associated with adverse pregnancy outcomes, including transmission of the organism to the newborn, in addition to cervical neoplasia and pelvic inflammatory disease. The most significant complication is increased risk of HIV transmission primarily because of the inflammatory response that compromises the mucosal barriers to HIV. *T. vaginalis* lacks a cyst stage, and the trophozoite stage is infective through sexual contact. The pear-shaped trophozoite assumes an ameboid form on contact with epithelial cells of the genital tract. Adhesion molecules, known as *lipoglycans*, mediate this binding. The organism is susceptible to rapid drying in the environment, but a few cases of nonsexual transmission have been reported.

In women, the infection is primarily localized to the vagina, resulting in itching and the production of a frothy, creamy, mucopurulent vaginal discharge as well as dysuria. About one third of infected women, however, are asymptomatic. A chronic state can develop, with mild symptoms of pruritus and scanty discharge. Women with chronic infection are often a major source of transmission to sexual partners. Men infected with *T. vaginalis* are usually asymptomatic and serve as carriers, although they can develop nonspecific urethritis with a milky discharge that lasts up to 4 weeks. *T. vaginalis* is now considered an important cause of nongonococcal urethritis. Long-term immunity is not developed after an acute infection, and reinfection can occur.

The diagnosis in women is typically made by finding the trophozoite in vaginal discharge or occasionally in urine; in men, the trophozoite is seen in urine or prostatic secretions. The organism is 5 to 18 μm in diameter and has four anterior flagella and an **undulating membrane** that extends one half the length of the body. In a wet mount, the trophozoite has a characteristic jerky motility, and the motion of the flagella and undulating membrane may be seen. The preparation should be examined

immediately because the organism loses viability quickly. In a Giemsa-stained preparation, the pear-shaped organism shows the presence of an axostyle extending the length of the organism, a single nucleus near the anterior end, and chromatic granules extending the length of the axostyle. Fluorescent staining of exudates using acridine orange will demonstrate a typical fluorescent morphology with a yellow to green nucleus. Although most clinicians rely on wet mount preparations, the method has relatively low sensitivity (30% to 70%).

Until the advent of molecular methods, culture of the organism from exudate was considered the most sensitive and specific method of detection, although an inoculum of 300 to 500 organisms is necessary. Commercial systems using a plastic pouch containing culture medium are available for detection of the organisms. The specimen should be inoculated into the pouch within 30 minutes of collection to ensure optimal viability of the organism. Culture specimens should be held for up to 5 days and then examined microscopically through the pouch. PCR methods and antigen detection assays are also commercially available. Rapid antigen detection tests are based on immunochromatographic EIA procedures using monoclonal antibodies. These tests have relatively high sensitivity compared with examination of a wet preparation and often test for more than one agent of vaginal infection. PCR tests are especially useful in detecting infection in asymptomatic men. Infections are usually treated with metronidazole. Treatment of sexual partners is suggested to obtain optimal cure and prevent reinfection.

Nonpathogenic intestinal flagellates

Chilomastix mesnili and *Pentatrichomonas* (*Trichomonas*) *hominis* are intestinal nonpathogens that must be differentiated from pathogenic flagellates. *C. mesnili* trophozoites are pear shaped and approximately 10 to 20 μm long $\times$ 3 to 10 μm wide. The cytostome and nucleus are prominent in the anterior of the organism, and a spiral groove encircles the body of the organism. The nucleus has a small central karyosome and is surrounded by fibrils that curl around the cytostome to give a so-called “shepherd’s crook” appearance. The cytostome is elongated and rounded at the anterior and posterior. The cyst of *C. mesnili*, which measures 6 to 10 μm , is lemon shaped and has an anterior nipple. The nucleus, cytostome, and curved fibrils are visible in a stained smear (Fig. 28.22).

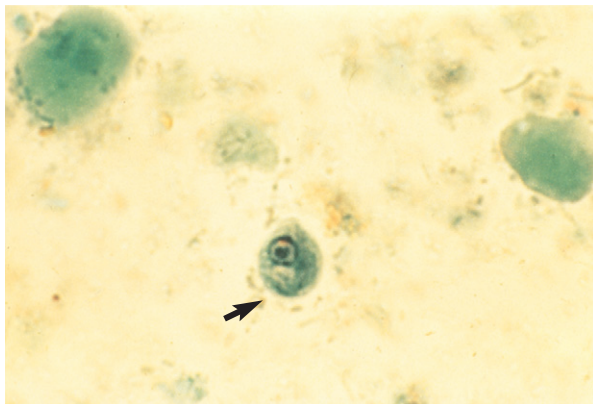


Fig. 28.22 *Chilomastix mesnili* cyst, identified by the arrow (trichrome stain, $\times 1000$).

The *P. hominis* trophozoite is 6 to 14 μm long, with a prominent axostyle extending through the posterior of the organism, four anterior flagella, and an oval nucleus with a small karyosome. The undulating membrane extends the length of the organism and is joined to the body along the costa. Trophozoites stain weakly, making them difficult to detect on stained smears. *Enteromonas hominis* and *Retortamonas intestinalis* are two additional nonpathogenic flagellates occasionally found in clinical specimens.

Case check 28.1

The intermittent bouts of diarrhea in the young patient in the Case in Point could be caused by any organism—viral, bacterial, or parasitic. The culture results were negative, so that would seemingly rule out a bacterial cause. Viral agents are not routinely cultured, nor would we expect viral causes to extend for such a long period. Stools from patients with parasitic infections caused by *G. duodenalis* are usually characterized as having a pale, frothy, gassy, and greasy appearance. Stools from patients with symptomatic amebiasis caused by *E. histolytica* often demonstrate blood. Neither organism is associated with *pica* (eating dirt or other nonfood material). In general, infections with protozoa do not cause eosinophilia.

Blood and tissue flagellates

The hemoflagellates in the genera *Leishmania* and *Trypanosoma* differ in several ways from the intestinal flagellates. First, they are transmitted by insect **vectors**, organisms that can transmit disease-causing agents. These vectors are necessary for completion of the life cycle. Second, these organisms have different life cycle stages for diagnosis. Fig. 28.23 shows the four life cycle stages of the hemoflagellates. The trypomastigote and amastigote are the diagnostic stages found in humans. The amastigote is an obligate intracellular organism, 2 to 5 μm in diameter, found within macrophages and liver, spleen, or bone marrow cells in diseases caused by *Leishmania* spp. In addition, the amastigote stage may be seen in cardiac or GI cells of patients infected with *Trypanosoma cruzi*. The trypomastigote, a flagellated form measuring 15 to 20 μm in size, is found in the blood, lymphatic fluid, and CSF of patients infected by *Trypanosoma* spp. The epimastigote and promastigote stages are seen in the insect vectors.

Leishmania

The genus *Leishmania* contains several complexes of at least 20 species that cause disease in humans: *Leishmania tropica*, *Leishmania mexicana*, *Leishmania braziliensis*, and *Leishmania donovani* complexes. Leishmaniasis is a zoonotic infection in which dogs and rodents serve as the primary reservoir hosts for all species, and humans serve as an accidental host. The insect vectors are sand flies of the genera *Phlebotomus* and *Lutzomyia*.

Clinical infections

Clinical infections range from single, self-healing skin ulcers to visceral disease affecting multiple organs. *L. tropica* complex, the cause of cutaneous leishmaniasis, or Oriental sore, is found primarily in the Far East and North and Central Africa. The condition is characterized by the presence of a crusted

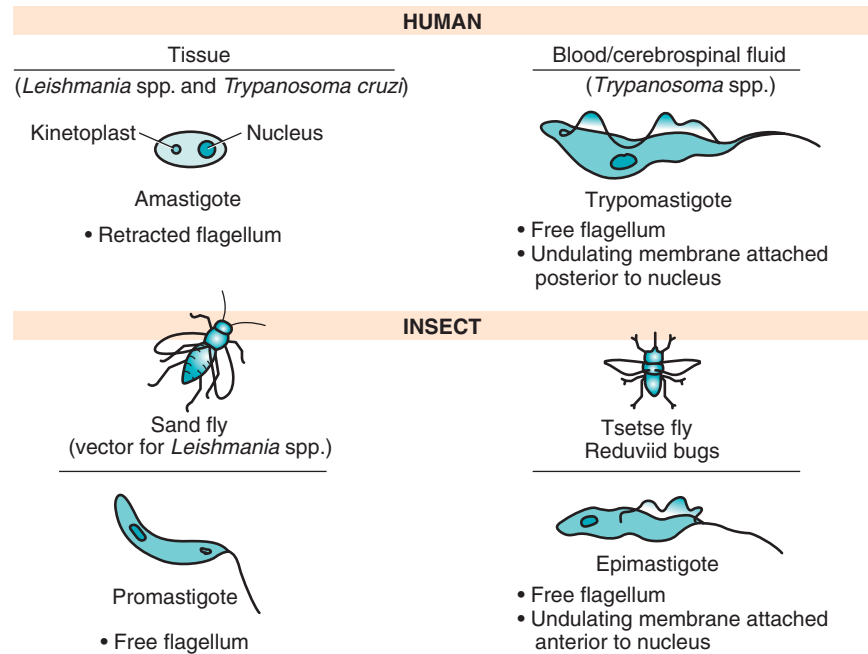


Fig. 28.23 Life cycle stages of the blood and tissue flagellates.

circular lesion on any exposed body surface, especially the face and extremities. The lesion begins as a small, red papule at the site of the insect bite and progresses to a lesion with an elevated indurated margin that may reach 8 cm. The lesion resolves in several months and provides incomplete immunity against future infection. *L. mexicana* complex, the cause of New World cutaneous leishmaniasis, is found in South and Central America. Another form of the disease, chiclero ulcer—often associated with *L. mexicana* complex—is characterized by lesions on the ear. The infection is self-limiting and does not invade mucosal surfaces, but secondary bacterial infection can occur.

L. braziliensis complex is the causative agent of mucocutaneous leishmaniasis, or espundia. This infection manifests itself as an initial lesion that may increase in size, invading and destroying the mucosal surfaces of the nose and mouth. It may also destroy cartilage, leaving the patient with significant disfigurement. *L. braziliensis* complex is found primarily in Mexico and Central and South America.

The most severe infection, visceral leishmaniasis, or kala-azar, is endemic in parts of South America, Africa, southern Europe, and Asia, and it commonly affects children. The causative agents are organisms of the *L. donovani* complex. In this disease, organisms spread through the lymphatics and invade organs of the reticuloendothelial system, including the liver, spleen, lymph nodes, and bone marrow. Patients with kala-azar exhibit malaise, anorexia, weight loss, headache, and fever. In addition, they may show splenomegaly and hepatomegaly with elevated liver enzyme levels, hypogammaglobulinemia, and hypoproteinemia. *Leishmania*-containing macrophages invade the bone marrow. The kidneys and heart may also be affected.

A few patients experience a nodular or macular rash, known as *post-kala-azar dermal leishmaniasis*, on the face, trunk, and limbs months to years after treatment. If untreated, visceral leishmaniasis is often fatal within 2 years. Cases of cutaneous leishmaniasis and visceral leishmaniasis have been

seen in several military personnel serving in Middle Eastern countries, such as Iraq and Afghanistan. Visceral leishmaniasis has also been reported to be transmitted through solid organ transplants. Previously, standard therapy for all leishmanial infections was pentavalent antimony compounds. However, liposomal amphotericin B and paromomycin were used in the past few years. A new drug, miltefosine, has been successful in treating infections.

Life cycle

Fig. 28.24 shows the generalized life cycle for *Leishmania* spp. When the female insect takes a blood meal, she ingests the amastigote stage. The parasite develops as a promastigote in the gut of the insect and migrates to the salivary glands when mature. The promastigote is transmitted to humans through the salivary glands of the insect when it takes a blood meal. The promastigote is inoculated into the dermal-epidermal junction and taken up via receptor-mediated phagocytosis by a macrophage. Within the macrophage, it converts to the amastigote stage and multiplies within the cell. When the cell ruptures, amastigotes are released and invade other macrophages. Parasite and immune cell interactions may lead to an inflammatory response that controls the parasite but also causes tissue damage.

Laboratory diagnosis

The amastigote is the diagnostic stage in humans. It is a small intracellular stage found in macrophages or histiocytes around the periphery of the skin lesions (*L. tropica* or *L. braziliensis*) or within cells of a bone marrow aspirate or liver or spleen biopsy specimen (*L. donovani*). Wright staining of bone marrow aspirate reveals an oval organism 2 to 5 μm in diameter, with pale blue cytoplasm, a large red nucleus, and a rodlike **kinetoplast** (a structure rich in DNA associated with the basal body near the base of the flagellum) within the cytoplasm (Fig. 28.25). The amastigote has also been reported extracellularly in cases of cutaneous leishmaniasis.

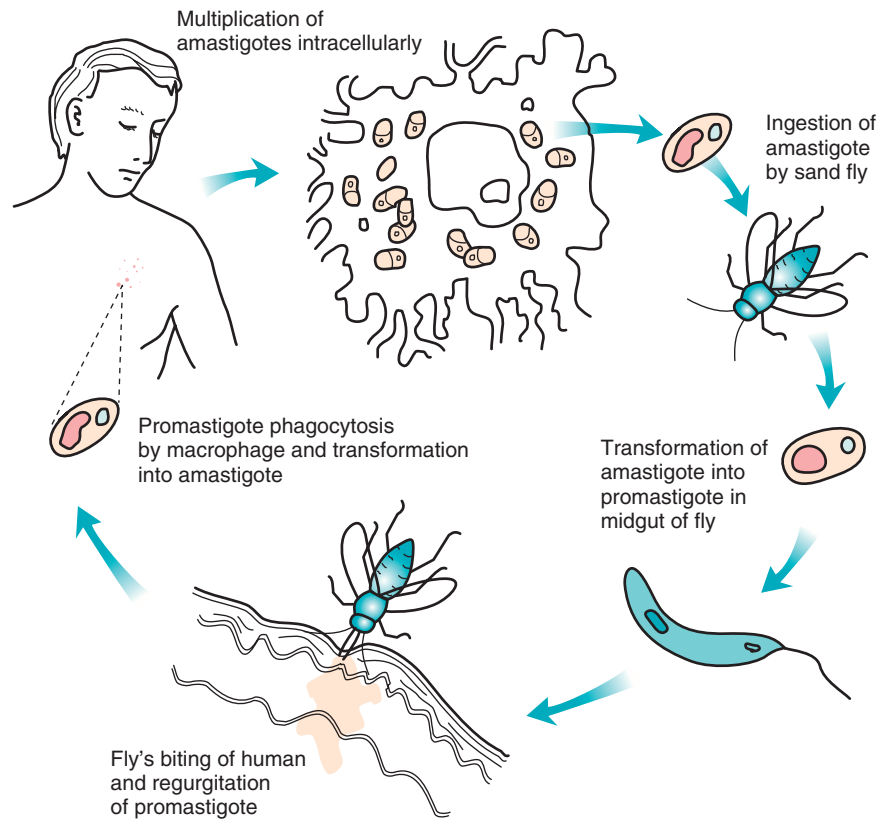


Fig. 28.24 Life cycle of *Leishmania* spp.

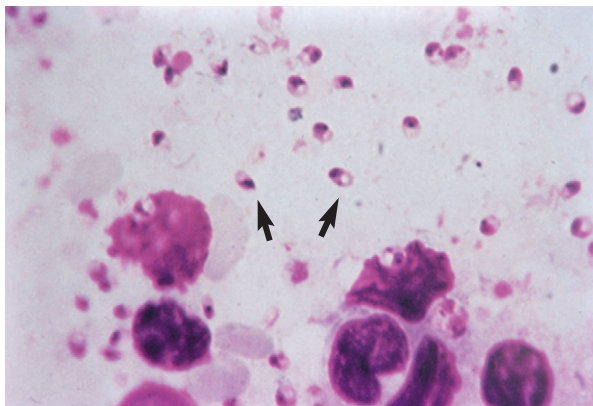


Fig. 28.25 Amastigotes of *Leishmania* spp., identified by arrows (Giemsa stain, $\times 1000$).

Promastigotes (the insect form of the parasite) have been identified in some patient samples. Serologic tests, such as direct agglutination and indirect fluorescent assays, can be used to detect antibodies in individuals from nonendemic areas.

Trypanosoma

Trypanosomes are blood and CSF flagellates that require an insect vector. *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense* are the causative agents of human African trypanosomiasis, commonly called *sleeping sickness*, which is seen primarily in Central Africa. The tsetse fly (genus *Glossina*) serves as the insect vector. West African sleeping sickness, caused by *T. brucei gambiense*, is the more common, milder,

and more chronic of the two diseases. A long asymptomatic stage is followed by death years later. East African sleeping sickness, caused by *T. brucei rhodesiense*, is characterized by a rapid course, often resulting in death within 6 months after the onset of symptoms. East African sleeping sickness is a zoonosis, with game animals serving as important reservoirs of *T. brucei rhodesiense*. Humans are the main reservoir for *T. brucei gambiense*, but this species can sometimes be found in animals. *T. cruzi*, the agent of American trypanosomiasis or Chagas disease, is discussed in the next section.

Clinical infections

Initial symptoms of African sleeping sickness include a local inflammatory reaction with tenderness, edema, and erythema at the site of the insect bite. This occurs within 2 to 3 days and can last up to 4 weeks. During this time, the organisms are reproducing and beginning to enter the bloodstream. The first stage of the disease is the hemolymphatic phase, which occurs about 1 to 3 weeks after infection. Trypomastigotes enter the blood and lymphatics, and the patient experiences generalized symptoms, including fever, headache, joint and muscle pain, weakness, and lymphadenopathy. Enlargement of the lymph glands in the posterolateral triangle of the neck is known as the *Winterbottom sign*. Edema in the legs and arms and around the eyes is possible. While circulating, the organisms can resist antibody-mediated destruction as a result of continuous antigenic variation. A protective glycoprotein layer helps the organism resist direct lysis by complement. The second (meningoencephalitic) stage begins as the trypomastigotes invade the CNS; the patient develops severe headaches, mental dullness, and apathy, and may experience

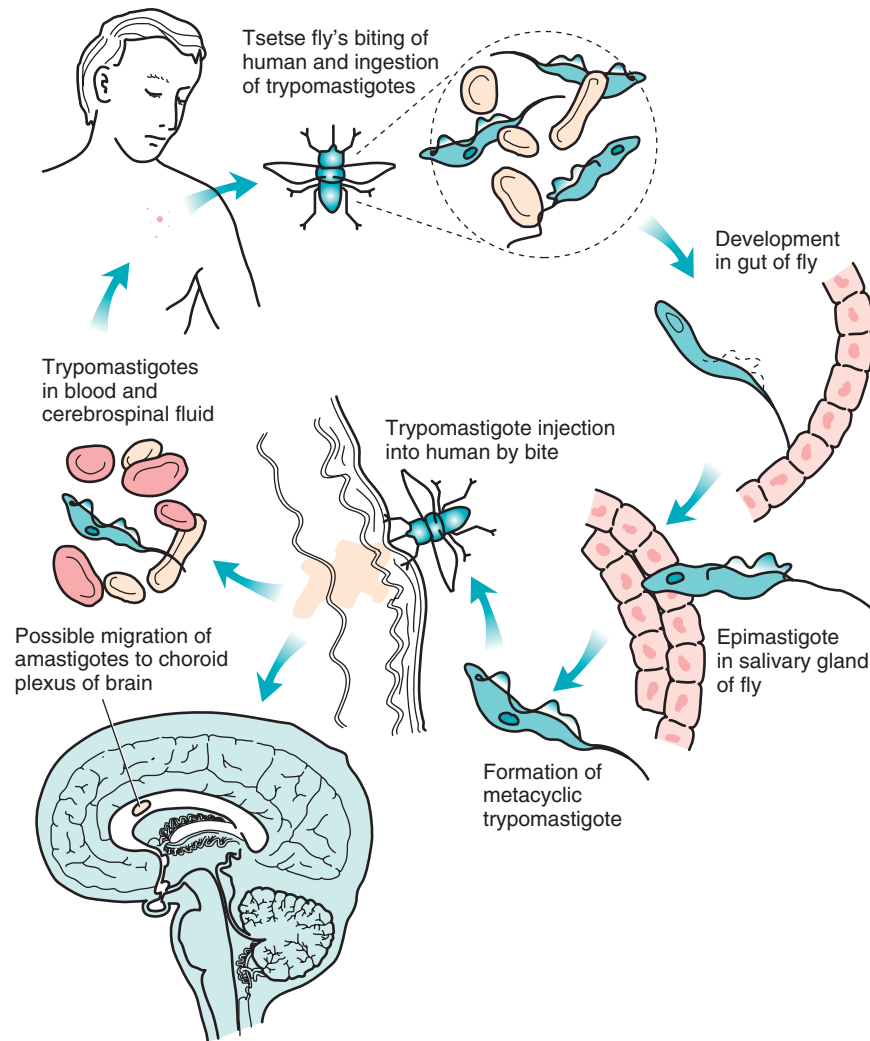


Fig. 28.26 Life cycle of the agents of sleeping sickness (*Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense*).

coordination problems, altered reflexes, and paralysis. Eventually, the patient has convulsions, lapses into a coma, and dies.

Life cycle

The tsetse fly is the biological vector for agents of sleeping sickness. Fig. 28.26 shows the generalized life cycle of these agents. The fly ingests the trypomastigote stage when it takes a blood meal from a human. The organisms migrate to the insect gut and multiply. The organisms later migrate back to the salivary gland where they transform into the epimastigote form. The epimastigotes multiply and develop into infective metacyclic trypomastigotes, which are transmitted to humans in saliva when the fly takes another blood meal. The trypomastigote form first circulates in the blood and lymphatic system, ultimately invading the CNS.

Laboratory diagnosis

The diagnostic stage in humans is the trypomastigote, which is usually seen in a Wright-stained blood smear, although wet films may be used to detect motile trypomastigotes. Blood concentration methods are often needed because of the small

number of organisms present. The organism can also be detected in lymphatic fluid and CSF. In infected individuals, CSF will often show increased numbers of WBCs (primarily lymphocytes) and elevated protein levels. The trypomastigote is 15 to 20 μm long, with a single large nucleus and a posterior kinetoplast to which is attached the flagellum of the undulating membrane (Fig. 28.27). *Trypanosoma* spp. cannot be differentiated on a blood smear; therefore the organism is reported as *Trypanosoma* sp. Final determination of species may be made on the basis of clinical symptoms and the geographic area.

The QBC method, which has been used in the detection of malarial parasites, has also been adapted for the detection of trypomastigotes. Field diagnosis of infection is important in endemic areas, and a serologic method using a card agglutination test for trypanosomiasis (CATT) and a micro-CATT have been developed. The test uses lyophilized *T. brucei gambiense* antigens to detect antibodies in blood. The test cannot distinguish current infection from past infection; therefore microscopy should be used to confirm the presence of the organisms in blood. It cannot be used as a test of cure because antibodies can persist for years after treatment. A latex agglutination test

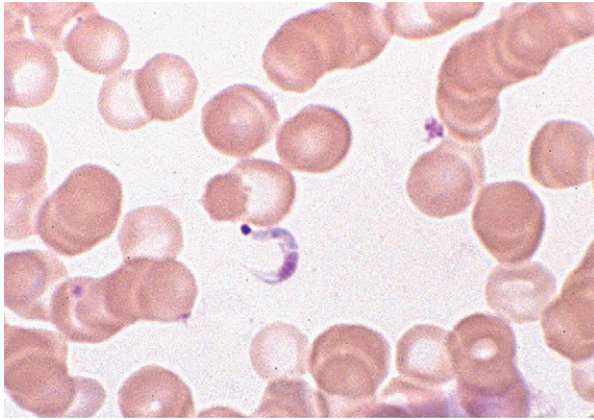


Fig. 28.27 *Trypanosoma* trypomastigote in a blood smear. The anterior flagellum is not visible in this organism. (Giemsa stain, $\times 1000$.)

that uses three surface antigens is considered more specific than the CATT. There is no comparable method for detecting *T. brucei rhodesiense*.

Trypanosoma cruzi

Chagas disease is a zoonotic infection found primarily in rural areas of Mexico and Central and South America. It is caused by the hemoflagellate *T. cruzi*, transmitted by the triatomid bug, also known as the *reduviid* or *kissing bug* (*Triatoma* sp. or *Panstrongylus* sp.). It has been estimated that up to 20 million people in these regions are infected. Reduviid bugs live in the mud walls or thatch walls of a dwelling during the day and come out at night to take a blood meal from the human inhabitants. Some animals, such as armadillos and opossums, may also be infected by the organism and serve as reservoirs. Sporadic cases of infection have been reported among nonimmigrants in the United States because several of the insect vectors exist there.

Although insect transmission is most common, the organism has been transmitted via blood transfusion (especially platelet concentrate), transplanted organs, and congenital infection. In endemic areas of the world, transmission via transfusion is relatively common, and in North America, several cases of transfusion-transmitted Chagas disease have been reported. Donors in these cases were asymptomatic and in the latent or chronic state of the disease. There are estimates that greater than 300,000 immigrants living in the United States are chronically infected and could be capable of transmitting Chagas disease through transfusions or solid organ transplantations. Screening of blood donors for antibodies to *T. cruzi* in some countries of Central and South America has decreased the rate of transfusion-related transmission. Screening tests for blood donors in the United States have been implemented.

Clinical infections

Infections may be symptomatic or asymptomatic. The infection is divided into three phases: acute, intermediate (latent), and chronic. After the insect bites, there is an incubation period that ranges from 2 to 4 weeks. As the organisms enter the blood, the acute phase begins and lasts 4 to 8 weeks. Individuals in the acute phase may be asymptomatic or have local symptoms, including the presence of a *chagoma* (ulcerative skin lesion at the site of the insect bite) or unilateral

edema around the eye (Romaña sign) if the bite is near the ocular conjunctiva. Systemic manifestations include fever, lymphadenitis, hepatosplenomegaly, malaise, muscular pains, and diarrhea and vomiting. Adults often have a milder form of the disease, and the most severe form of acute infection usually occurs in children. This may manifest itself as myocarditis or meningoencephalitis. If untreated during the acute phase, the patient will most likely remain chronically infected.

The latent period develops as the trypomastigotes disappear from the circulation and invade the cells of the cardiac or GI system. The latent phase can last 10 to 40 years after infection, but only about 30% to 40% of patients develop the chronic form of Chagas disease—primarily cardiomyopathy. Chronic myocarditis develops with damage to all heart chambers and the conduction system. This may progress to tachycardia and eventually congestive heart failure. In some patients, the chronic form may manifest itself as megacolon, megastomach, or megaesophagus, which results in difficulty swallowing and disturbances to intestinal motility. Damage to the organs is caused by a combination of continuous low-level parasitemia and immune-mediated tissue damage, including high levels of TNF- α and IFN- γ . Polyclonal B-cell activation in the acute phase produces antibodies that have a weak affinity for the organism and may cross-react with heart tissue. Controversy exists about the role of these autoantibodies in tissue damage. When the patient enters the chronic stage of the disease, there are periods of intermittent parasitemia of trypomastigotes, during which time the organism may be transmitted.

Life cycle

Transmission of the organism to humans occurs when the insect vector defecates in the area surrounding its bite site. Metacyclic trypomastigotes in the feces are scratched into the bite site and invade the bloodstream. The trypomastigotes circulate in the bloodstream and eventually enter the cells, where they transform into amastigotes. Cells of the cardiac muscle and skeletal muscle are most commonly infected. Within the cell, the amastigote multiplies and causes the cell to rupture. Free amastigotes then invade other cells. A few of the released amastigotes will transform into trypomastigotes in the bloodstream and can be ingested by the insect to continue the life cycle. [Fig. 28.28](#) shows the life cycle of *T. cruzi*.

Laboratory diagnosis

The primary diagnostic stage in blood during acute infection is the trypomastigote. It is an elongated structure 15 to 20 μm long that often appears in a C or U shape. Like the other trypomastigotes, it shows a single, large-nucleus midbody; a single anterior flagellum; and a posterior kinetoplast to which the undulating membrane is attached. In most Wright-stained smears, the nucleus and kinetoplast and parts of the undulating membrane are well stained, but only the suggestion of a flagellum can be seen at the anterior end. In the chronic stage, the organism can be seen as an amastigote in a cardiac or other tissue biopsy specimen. The morphology of all trypomastigotes is similar (see [Fig. 28.27](#)); therefore the complete patient history must be obtained to determine the species present. If the history is not available, then the organisms are reported as trypomastigotes of *Trypanosoma* spp. Diagnosis of chronic infection with *T. cruzi* is based on patient history, clinical symptoms, and

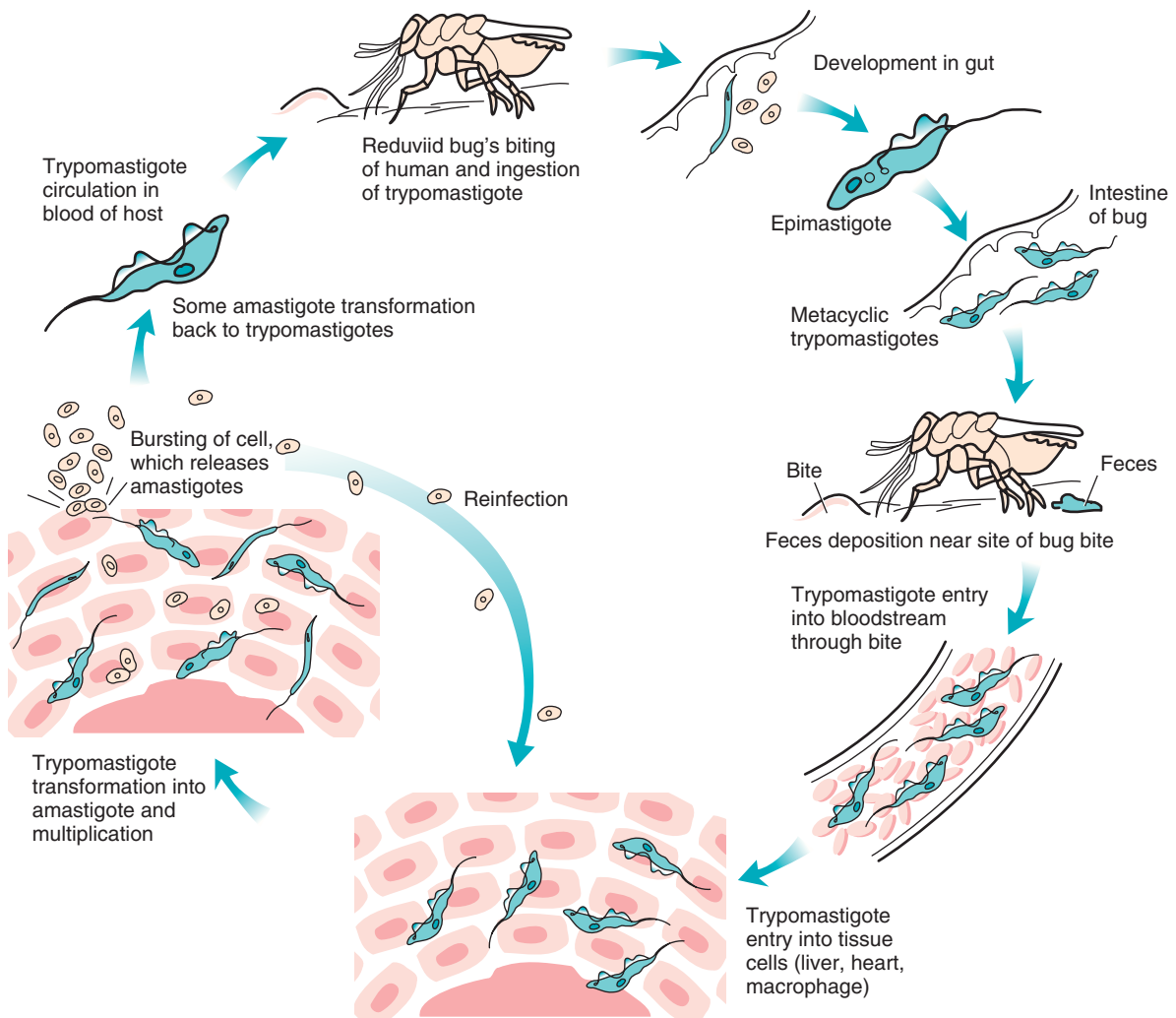


Fig. 28.28 Life cycle of *Trypanosoma cruzi*.

presence of IgG antibodies to the organism. Serologic testing for antibodies to *T. cruzi* is also used in screening blood donors in the United States and other countries.

Xenodiagnosis is used in Central and South America as a diagnostic method. A laboratory-raised triatomid bug is allowed to feed on patients suspected of having *T. cruzi* infection. The insect's feces are examined on a regular basis for the presence of the parasite. Presence of the parasite in the insect's feces indicates that the patient was infected. This method is most helpful for diagnosing infections in the chronic stage, when fewer parasites are present. Rapid diagnostic tests (RDTs) using whole blood are under evaluation for testing individuals in remote areas.

Apicomplexa

The phylum Apicomplexa includes blood and tissue parasites. This group of organisms shows a diversity of morphology and transmission methods and can infect many different body sites. The life cycles are complex, characterized by sexual reproduction and **asexual reproduction** (reproduction when offspring arise from a single parent) phases. In addition, some may require an insect vector or intermediate host for completion of the life cycle. Humans can serve as **definitive hosts**

when **sexual reproduction** (offspring arise from the exchange of genetic material between two parents) occurs in human tissues and as intermediate hosts when asexual reproduction occurs.

Plasmodium

Plasmodium spp., which cause malaria, remain endemic throughout the world in tropical and subtropical countries, with almost one half of the world's population residing in an endemic area. *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae*, and *Plasmodium falciparum* are the causative agents of human malaria. A fifth species, *Plasmodium knowlesi*, which originated in simian species, is now associated with cases of human malaria and has been reported in most countries of Southeast Asia. Along with schistosomiasis and amebiasis, malaria is a major cause of death in people in underdeveloped countries. In 2020, there were an estimated 241 million cases worldwide with 627,000 deaths. The majority of infections occur in sub-Saharan Africa, where *P. falciparum* is the predominant species. Children under 5 years of age are the most at risk and accounted for 77% of malaria deaths in 2020. In the United States, approximately 2000 cases are reported annually, with *P. falciparum* being the causative agent in more

than 50% of cases. Most cases are seen in persons who have traveled to endemic areas.

P. vivax has the widest geographic distribution and is the species most likely to be found in temperate climates. *P. vivax* and *P. falciparum* cause more than 95% of infections. *P. ovale* is primarily confined to western parts of Africa; *P. falciparum* and *P. malariae* have similar distributions throughout Africa and in tropical countries. In general, infections caused by *P. vivax*, *P. ovale*, and *P. malariae* are less severe than those caused by *P. falciparum*.

Clinical infections

Although malaria is usually transmitted through the bite of an anopheline mosquito, transmission through blood transfusion and infected needles has been reported. Transplacental infection also has been documented. In these cases, the organism is sequestered in the placenta and can compromise fetal development because it affects transplacental transport of nutrients.

The malarial paroxysm, which begins 10 to 15 days after the bite of an infected mosquito, is associated with the growth of the parasites in RBCs and release of high levels of cytokines. Symptoms are linked to the rupture of RBCs and release of **merozoites** (parasitic forms produced from multiple fission), malarial metabolites, and endotoxin-like substances into the bloodstream.

The malarial paroxysm has three phases. The prodromal phase involves headache, bone pain, nausea, and/or flulike symptoms. In the first phase the patient experiences a shaking chill lasting 15 to 60 minutes. The second phase is characterized by a fever up to 40° C along with headache, myalgia, and nausea lasting 2 to 4 hours. In the third and final phase the fever finally breaks. Over the course of 2 to 4 hours, the patient begins to sweat profusely and is left exhausted and sleepy. This cycle repeats itself at regular intervals, depending on the species of malarial organism present, as parasites exit infected RBCs. The anemia seen in malaria is the result of lysis of erythrocytes, removal of the infected cells by splenic macrophages, and in some cases immune clearance of erythrocytes coated with immune complexes of malarial antigens and antibodies. In small children and in those with heavy infections, severe anemia can develop.

Long-term infection with any malarial organism may result in damage to the liver and spleen caused by deposits of malarial pigment (hemozoin). *P. vivax* and *P. ovale* infections generally do not have the range of complications seen with other species. Because the organism invades reticulocytes, there is generally low-level parasitemia (2% to 5%). However, the persistence of hypnozoites that remain dormant in the liver can lead to relapses within 3 years of initial exposure. *P. malariae* infections can lead to nephrotic syndrome, which arises from the deposition of circulating immune complexes of malarial antigens and antibodies on the basement membrane of the glomeruli, causing an autoimmune-like reaction. This organism has the most chronic course, and recrudescence occurs even decades after the initial infection.

The most severe form of malaria is caused by *P. falciparum*. Up to 50% of erythrocytes may be infected, and the primary complication is development of cerebral malaria. Between 20% and 50% of deaths caused by *P. falciparum* are the result of CNS complications. The parasite-infected RBCs demonstrate

sticky knobs that mediate adhesion to the endothelial cells of the capillary walls. The parasite protein *P. falciparum* erythrocyte membrane protein 1 is involved in mediating this attachment. In addition, complement receptor 1 on the surface of erythrocytes may help infected cells bind with other erythrocytes to form rosettes that can obstruct small capillaries. Blood flow is slowed in the microcirculation, reducing oxygen delivery to the tissues, with resultant tissue anoxia. The patient has severe headaches, may be confused, and ultimately lapses into a coma.

A second but less common complication of infection with *P. falciparum* is **blackwater fever**, a condition characterized by hemoglobinuria (hence the term *blackwater*). It usually develops in patients with repeated infections and those undergoing quinine therapy. Blackwater fever may be mediated by an antigen-antibody reaction caused by the development of an autoantibody to the RBCs. The black appearance of the urine is the result of massive intravascular hemolysis and resulting hemoglobinuria that appears black in acid urine. In addition, the high-level parasitemia that often accompanies *P. falciparum* infection can lead to anemia and deposits of malarial pigment in some organs, such as the spleen. Renal complications, such as nephrotic syndrome or even renal failure, can occur with *P. falciparum* infection as a result of tubular necrosis brought about by tissue anoxia.

Although complete immunity to malaria does not exist, individuals in endemic areas develop antibodies against the asexual stages and merozoites, which helps reduce the parasite load and severity of illness. Merozoite-specific antibodies may help block invasion of the RBCs or enhance opsonization and induce phagocytosis by macrophages. In addition, reports of antibodies directed against **sporozoites** and **gametes** (sexual reproductive cells that unite with another cell to form a zygote) indicate that these also may help reduce the rate of infection and the severity of illness. Patients with hemoglobinopathies, such as sickle cell disease, hemoglobin C disease, and glucose-6-phosphate dehydrogenase deficiency, are somewhat protected against severe malaria because the parasite cannot replicate or, in some cases, exist in these RBCs.

Treatment

Chloroquine remains the primary drug for prophylaxis and treatment of malaria. It is effective against all asexual stages of malarial organisms and all gametocytes, except those of *P. falciparum*. Many strains of *P. falciparum* have become resistant to the drug. Recent reports indicate that strains of *P. vivax* in Southeast Asia and Papua New Guinea also show diminished response to treatment with chloroquine.

P. falciparum has also demonstrated resistance to sulfadoxine-pyrimethamine (Fansidar) and mefloquine (Lariam) in parts of South America, Southeast Asia, and Africa. Doxycycline, atovaquone-proguanil (Malarone), or quinine use has been reestablished in some cases of multidrug-resistant *P. falciparum*. Chloroquine-resistant *P. vivax* may be treated with mefloquine or quinine sulfate with doxycycline. Primaquine phosphate, which is effective against hypnozoites that persist in the liver, is used to treat individuals infected with *P. vivax* and *P. ovale* to prevent relapses of these infections. There is also evidence that some strains of *P. vivax* in the geographic areas of chloroquine resistance may be refractory to primaquine. Artemisinin-based drugs have been used in

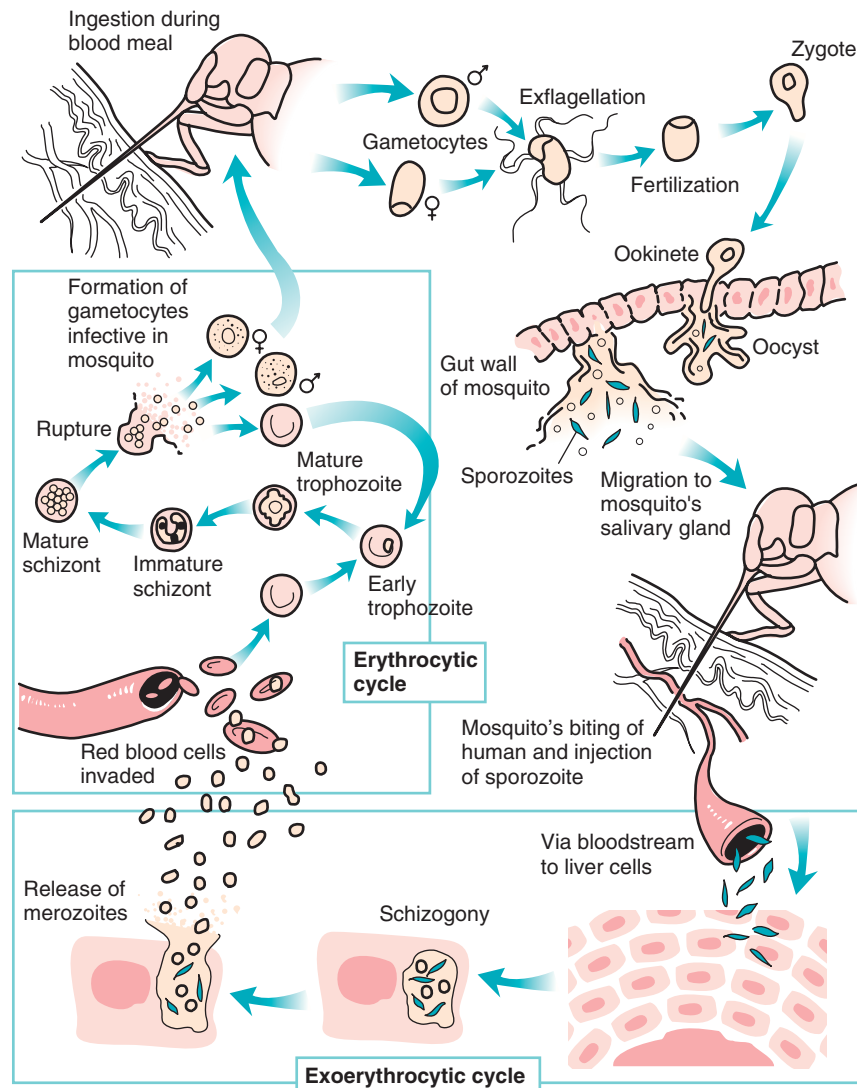


Fig. 28.29 Life cycle of *Plasmodium* spp.

combination with classic drugs in areas in which highly resistant strains of *P. falciparum* have emerged.

The level of parasitemia should be calculated as a percentage before and during treatment to follow effectiveness of treatment. In cases of high-level parasitemia, an RBC exchange may be used before treatment to decrease the level of parasitemia and increase effectiveness of the drug.

Life cycle

The life cycle of *Plasmodium* involves sexual reproduction (**sporogony**) and asexual reproduction (**schizogony**), as shown in Fig. 28.29. Sporogony is the formation of oocysts containing sporozoites that results from the division of a zygote. Schizogony produces merozoites by the process of multiple fission. The female *Anopheles* mosquito, in which sexual reproduction occurs, serves as the biological vector and definitive host. Asexual reproduction, which occurs in humans, has an **exoerythrocytic phase** that takes place in the liver and an **erythrocytic phase** that takes place in erythrocytes.

In human infections, the earliest stage is the ring-form trophozoite (Fig. 28.30), in which the organism has a prominent, red-to-purple chromatin dot and a small blue ring

of cytoplasm surrounding a vacuole (in a Giemsa-stained blood smear). The growing trophozoite is characterized by an increase in the amount of cytoplasm, the disappearance of the vacuole, and the appearance of malarial pigment in the organism's cytoplasm. The immature **schizont** (multinucleate stage) is characterized by a splitting of the chromatin mass. The mature schizont contains merozoites, which are individual chromatin masses, each surrounded by cytoplasm. Microgametocytes (male) have pale blue cytoplasm and a diffuse chromatin mass that stains pale pink to purple. The chromatin may be surrounded by a clear halo. Macrogametocytes (female) show a well-defined, compact chromatin mass that stains dark pink; the cytoplasm stains a darker blue than in microgametocytes. The chromatin mass often is set eccentrically in the organism. Pigment is distributed throughout the cytoplasm except in *P. falciparum*, in which it is often clumped near the chromatin mass.

Exoerythrocytic phase

Humans serve as intermediate hosts and acquire the infection when the female mosquito takes a blood meal and injects the infective sporozoites with salivary secretions. The sporozoites

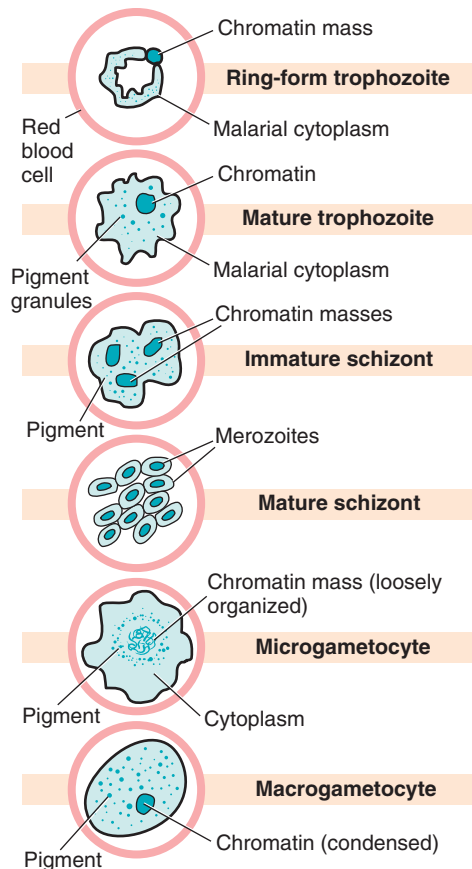


Fig. 28.30 Life cycle stages of *Plasmodium* spp.

enter the human circulation and take approximately 60 minutes to reach the liver, where they begin the exoerythrocytic phase by penetrating parenchymal cells. Maturation through the trophozoite and schizont phases results in the production of hepatic merozoites. Each schizont produces a large number of merozoites. The release of mature merozoites from liver cells and invasion of RBCs signal the beginning of the erythrocytic phase. Generally, only one cycle of merozoite production occurs in the liver before RBCs are invaded. *P. vivax* and *P. ovale*, however, may persist in the liver in a dormant stage known as *hypnozoites*, which accounts for the relapse (recurrence) of the disease within 1 to 3 years after the primary infection. Neither *P. malariae* nor *P. falciparum* has a persistent liver phase, although recrudescence in untreated individuals with either of these organisms may be the result of a continued subclinical erythrocytic infection.

Erythrocytic phase

Merozoites have structures and proteins (e.g., erythrocyte-binding antigen) that selectively adhere to receptors on the RBC membrane. *P. vivax* and *P. knowlesi* use antigens of the Duffy blood group as receptors for attachment to and internalization into the RBCs, whereas *P. falciparum* may simply attach to receptors that are integral parts of the RBC membrane itself. Organisms may evade antibody recognition by varying their antigenic makeup. When merozoites have attached, endocytic invagination of the RBC membrane allows the organism to enter the RBC within a vacuole.

Once inside the RBC, the organism feeds on hemoglobin and initiates the erythrocytic phase. Malarial pigment, which

is composed of iron deposits, is formed in the growing trophozoite as a result of incomplete metabolism of hemoglobin. Once the organism has reached the mature trophozoite stage, the chromatin begins to divide (developing schizont). When the chromatin split has been completed (mature schizont), each chromatin mass is surrounded by its own small amount of malarial cytoplasm (merozoite). Each malarial species has a typical number of merozoites, which may be used as an identifying characteristic. When the RBC ruptures, merozoites are released to invade other RBCs. At this point, two outcomes are possible. One is that the merozoite enters a cell and repeats development into a schizont, and the other is that it enters a cell and develops into one of the sexual stages, the microgametocyte or macrogametocyte.

Sexual phase

Sporogony, which takes place in the mosquito, results in the production of sporozoites infective for humans. Both the microgametocyte and the macrogametocyte are infective for the female mosquito when she takes a blood meal. In the insect's stomach, exflagellation by the male microgamete and subsequent fertilization of the female macrogamete result in formation of an ookinete that migrates through the gut wall and forms an oocyst on the exterior gut wall. Sporozoites are produced within the oocyst. Mature sporozoites are released into the body cavity of the mosquito and migrate to the salivary glands. The female then injects sporozoites into a human as she takes her blood meal.

Laboratory diagnosis

History of travel to an endemic area and the presence of classic clinical symptoms, including the malarial paroxysm of fever and chills, should alert a health care provider to request Giemsa-stained thick and thin smears for malaria. Examination of these blood smears remains the classic method of diagnosing malaria. Laboratory identification of malarial species involves examination of the morphology of infected RBCs and characteristic morphology of the parasite. The thick smear is used to detect malarial parasites; however, distortions of parasite morphology and the lack of intact RBCs require that a thin smear be examined to identify the species. Trophozoites, schizonts, and gametocytes may be seen in the blood smear. It is recommended that at least 200 to 300 oil immersion fields be examined on either type of smear before the smear is considered negative. In addition, more than one set of smears should be made within a 36-hour period because one set is inadequate to completely rule out the presence of malarial organisms. Table 28.6 gives the general characteristics of trophozoites and schizonts of the human malarial species.

The QBC system using the fluorescent dye acridine orange is a sensitive method for demonstrating the presence of parasites; however, a thin smear must still be examined for definitive identification of *Plasmodium* spp. Nucleic acid amplification tests have been proposed as another method for diagnosing malaria. They have good sensitivity in detecting low levels of *P. falciparum* infection. However, the extended time and costly equipment required do not make the procedure cost-effective or efficient, especially for field work.

Several different immunoassays, collectively referred to as *RDTs*, use malarial antibody-impregnated dipsticks to

Table 28.6 Comparisons of malarial species

Plasmodium species	Red blood cell morphology	Trophozoite	No. of merozoites in schizont	Reproductive cycle (hours)	Other characteristics
<i>P. vivax</i>	Enlarged (to $\times 2$) Schüffner dots	Ameboid Large vacuoles Golden-brown pigment	12–24 Average: 16	48	Wide range of stages in peripheral blood
<i>P. malariae</i>	Normal size May have dark hue Ziemann dots (rare)	Compact May assume band form across cell Coarse, dark brown pigment	6–12; average, 8 with daisy petal-like arrangement around clumped pigment	72	Wide range of stages in peripheral blood
<i>P. ovale</i>	Enlarged, oval Fringed edge Schüffner dots	Compact Golden-brown pigment (resembles <i>P. vivax</i>)	6–14 Average: 8	48	Wide range of stages in peripheral blood
<i>P. falciparum</i>	Normal size Multiple infections	Small, delicate Double chromatin dots Appliqué forms Dark pigment	8–24 Average: 20–24	Irregular, 36–48	Crescent-shaped gametocyte Ring and gametocyte stages only in peripheral blood

detect *Plasmodium* infection. These use an antigen capture immunochromatographic principle to detect soluble proteins from malarial organisms in blood. Some tests use a monoclonal antibody directed against an antigen specific to *P. falciparum*, histidine-rich protein 2. Other tests use an antibody that detects a protein common to all species, parasite lactate dehydrogenase or *Plasmodium* aldolase. One commercial rapid detection kit is approved for use in the United States. Serologic tests for antibody to malaria are not useful in an endemic area but may be useful for diagnosis in those who have traveled to an endemic area and have clinical symptoms of malaria. Testing for the presence of hemozoin in blood is an emerging area of malaria diagnostics.

Plasmodium vivax

Plasmodium vivax has a *tertian* life cycle pattern; that is, it takes approximately 48 hours for the life cycle to be completed. The invasion of a new group of RBCs begins on the third day. *P. vivax* usually invades young RBCs (reticulocytes) and therefore is characterized by enlarged infected RBCs, often up to double the normal size. A fine pink stippling known as **Schüffner stippling** (or dots) may be present in the infected cell. The young trophozoite is characterized by its ameboid appearance; by maturity, it usually fills the RBC, and golden-brown malarial pigment is present. The mature schizont contains 12 to 24 merozoites, with an average of 16. Gametocytes are rounded and fill the cell. Macrogametocytes are often difficult to differentiate from mature trophozoites. Fig. 28.31 shows several stages of *P. vivax*.

Plasmodium malariae

Plasmodium malariae usually invades older RBCs, perhaps accounting for the occasional darker appearance of the invaded RBC. The life cycle is characterized as *quartan*, with reproduction occurring every 72 hours and invasion of new RBCs every fourth day. The trophozoite is compact and may assume a characteristic band appearance, in which it stretches across the diameter of the RBCs (Fig. 28.32A). Note the presence of dark, coarse, brown-to-black pigment in the band form. Occasionally, a few pink cytoplasmic dots, called

Ziemann dots, may be seen. The mature schizont contains 6 to 14 merozoites (see Fig. 28.32B), with an average of eight. Merozoites may be arranged in a characteristic “loose daisy petal” arrangement around the clumped pigment; however, they may also be randomly arranged.

Plasmodium ovale

Plasmodium ovale, the least commonly seen species, resembles *P. vivax*. In *P. ovale* infections, the RBC is enlarged and may assume an oval shape with fimbriated or fringelike edges. Schüffner dots are less commonly seen than with *P. vivax*. The parasite remains compact, has a golden-brown pigment, and has a range of 6 to 14 merozoites in the mature schizont. It also exhibits a tertian life cycle. Fig. 28.33A shows trophozoites of *P. ovale*. The Schüffner stippling in Fig. 28.33B has stained almost a bluish pink, but the compact organism and fimbriated cell are characteristic.

Plasmodium falciparum

Although identified as having a tertian life cycle, *P. falciparum* often demonstrates an asynchronous life cycle, with rupture of RBCs taking place at irregular intervals, ranging from 36 to 48 hours. The life cycle stages seen in peripheral blood are usually limited to the ring-form trophozoite and gametocyte. Other stages mature in the venules and capillaries of the major organs. *P. falciparum* invades RBCs of any age and, for this reason, often exhibits the highest level of parasitemia, reaching 50% in some cases. The ring forms of *P. falciparum* (Fig. 28.34A) are more delicate than those of other species and often have two chromatin dots. **Appliqué forms**, which are parasites at the edge of the RBC, and multiple ring forms in a single RBC are common. In this figure, the appliqué form is seen in the lower left and upper right of the photograph. Occasionally, small comma-shaped, red dots, referred to as **Maurer dots**, can be seen in the cytoplasm of infected cells. The mature trophozoite is small and compact and may have a dark brown pigment. The schizont (which is rarely seen in peripheral blood) has 8 to 24 merozoites, with an average of 20 to 24. Gametocytes have a characteristic banana or crescent shape (see Fig. 28.34B). *P. knowlesi* has early life cycle stages

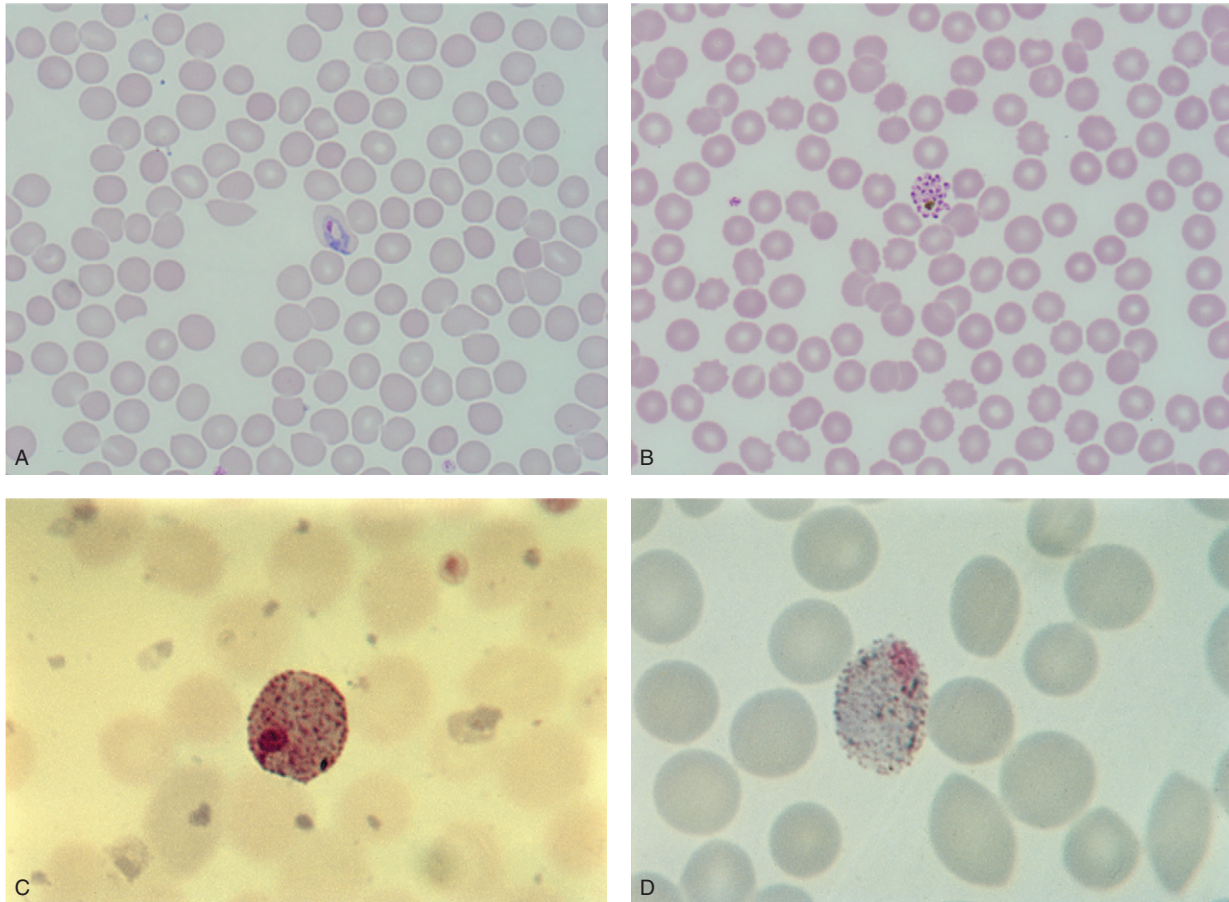


Fig. 28.31 A, *Plasmodium vivax* trophozoite. B, *P. vivax* mature schizont. C, *P. vivax* macrogametocyte. D, *P. vivax* microgametocyte (Giemsa stain, $\times 1000$).

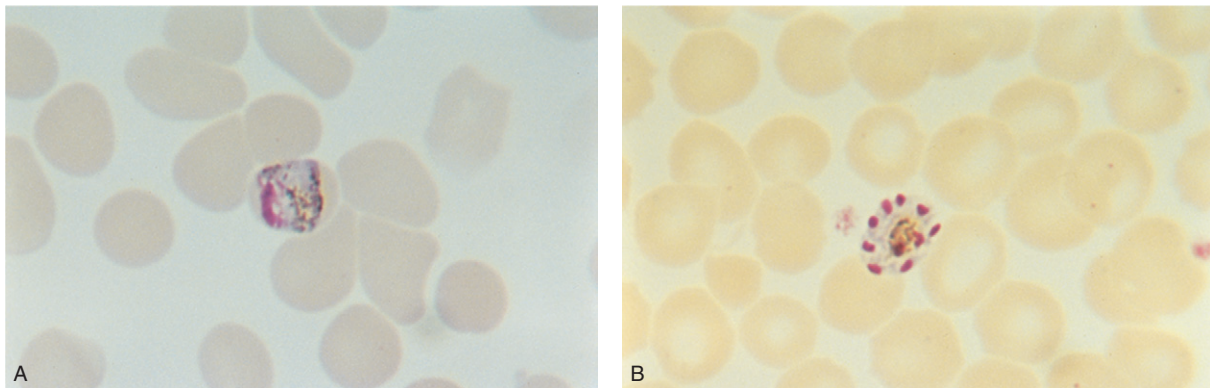


Fig. 28.32 A, *Plasmodium malariae* band form trophozoite. B, *P. malariae* schizont (Giemsa stain, $\times 1000$).

that resemble those of *P. falciparum* and mature trophozoites and gametocytes that resemble those of *P. malariae*. Therefore pertinent information, including travel history to an endemic area, is important in making a diagnosis.

Babesia spp.

Babesiosis is a zoonotic intraerythrocytic infection transmitted by the bite of a tick. *Babesia* spp. have been known to infect cattle and other vertebrates, but the first cases reported in humans occurred in the 1950s. These initial cases were limited to patients who had undergone splenectomy, but since then,

cases of babesiosis have been reported in patients who do not have asplenia. The hard tick (*Ixodes* spp.) serves as the vector, and white-footed mice and white-tailed deer are the reservoirs. There also have been reports indicating simultaneous transmission of *B. microti* and *Borrelia burgdorferi* (the causative agent of Lyme borreliosis) because these two organisms have the same tick vector. In the United States, most cases occur in the Northeast and upper Midwest. Patients often manifest clinical symptoms of one disease but show a concurrent increase and decrease in antibody titers to both *Babesia* spp. and *B. burgdorferi*.

There are over 100 species of *Babesia*, and several species have been reported to infect humans. *B. microti* in the United

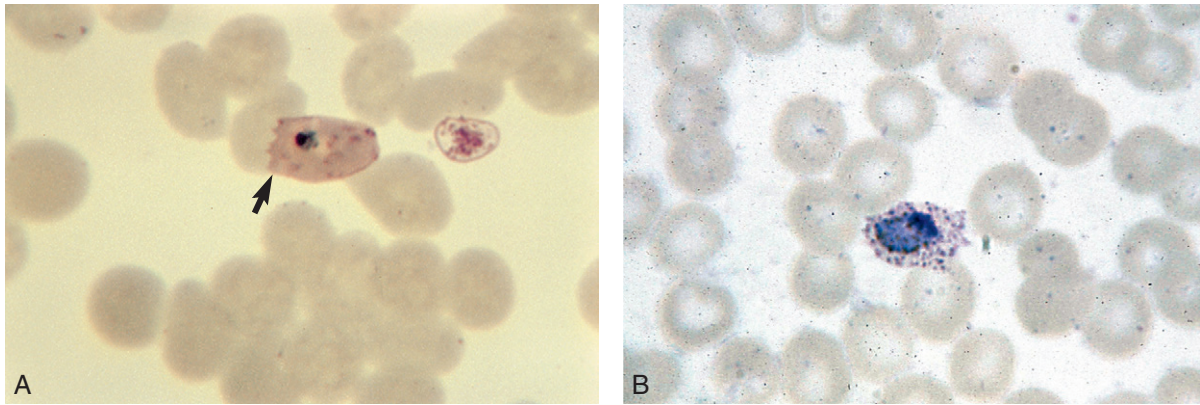


Fig. 28.33 **A**, *Plasmodium ovale* trophozoite. The arrow denotes the fimbriated edge of the red blood cell. **B**, Schüffner stippling of *P. ovale* clearly visible (Giemsa stain, $\times 1000$).

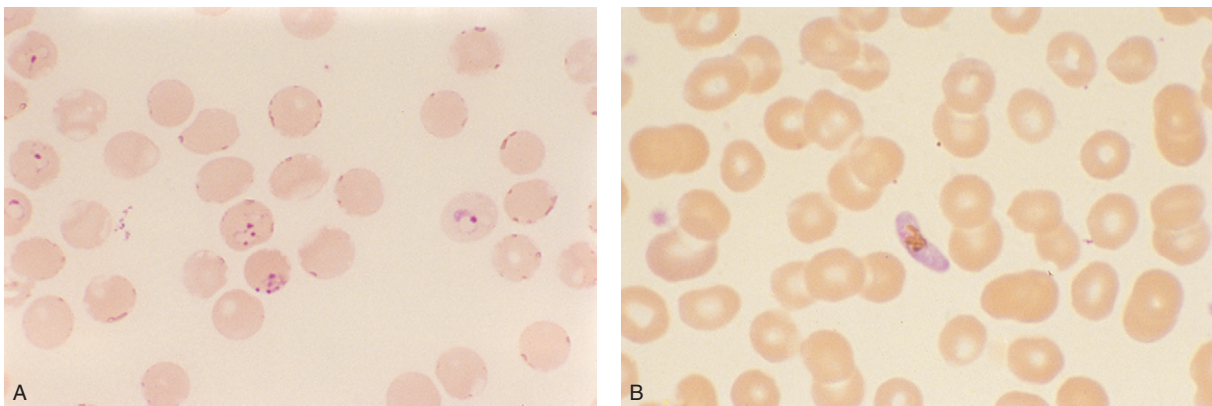


Fig. 28.34 **A**, *Plasmodium falciparum* ring-form trophozoites. **B**, *P. falciparum* gametocyte (Giemsa stain, $\times 1000$).

States and *B. divergens* in Europe are the most common. Several cases of *Babesia duncani* and *B. duncani*-like organisms have been reported in areas from the state of Washington to California. Sporadic cases in the states of Missouri, Kentucky, and Washington occurred with an organism that more closely resembles *Babesia divergens*, a parasite of cattle in Europe. As with malaria, cases of congenital babesiosis have been reported.

The incidence of babesiosis in the United States increased from 1,761 cases in 2013 to 2,418 in 2019. The disease is a potential threat to the U.S. blood supply. *B. microti* is the most common parasite transmitted in transfused blood. There were 159 cases of transfusion-transmitted babesiosis reported between 1979 and 2009, with 122 cases (77%) occurring between 2000 and 2009. Through 2014, the number increased to over 200 transfusion-transmitted cases. Most cases were linked to RBC components. Currently, there are no FDA-approved tests for screening donor blood units for antibodies to *Babesia* spp.

Clinical infections

Most cases of babesiosis occur from May to September, when outdoor activities peak and exposure to ticks is highest. Infection is often asymptomatic, but the very young, older adults, and immunosuppressed patients are at greatest risk of symptomatic infection. Symptoms are related to the asexual reproductive cycle, lysis of RBCs, and the level of parasitemia. The time from infection to development of

symptoms is 1 to 6 weeks. Patients with babesiosis may have malaria-like symptoms, such as malaise, fever, chills, sweating, and myalgia; however, many patients are asymptomatic. The fever is not cyclic as in malaria. Anemia may develop if hemolysis is severe or prolonged. The clinical course tends to be more severe or have complications if the patient has undergone splenectomy, is immunosuppressed, or is older. Complications may include respiratory, liver, or renal failure or disseminated intravascular coagulation. Treatment includes atovaquone plus azithromycin or clindamycin plus quinine. In very severe infections, exchange transfusion may be used.

Life cycle

As with *Plasmodium* spp., the life cycle of *Babesia* spp. has alternating sexual and asexual reproduction stages. Production of the infective stage (sexual reproduction) takes place in the tick. The zygote migrates to the salivary glands of the tick, where sporozoites are formed. These sporozoites enter vertebrates during a blood meal and infect erythrocytes by attaching to a sialoglycoprotein on the erythrocyte membrane. Inside the cell, they become trophozoites and divide by asexual reproduction (budding) to form pairs and tetrads. Unlike malaria, in which the reproductive life cycle in the RBC may take 48 to 72 hours, the reproduction time in *Babesia* infection is approximately 4 to 6 hours. The resulting merozoites are released to infect other RBCs, where they use merozoite

surface proteins to attach to glycophorin A and glycophorin B on the RBC membrane. Some merozoites differentiate into gametocytes (larger, more variably shaped), but this is not an easily identifiable stage from the trophozoite. One difference in this life cycle from that of malaria is the absence of the exoerythrocytic cycle in humans. Transovarian transmission can occur in the tick, allowing the life cycle to persist without an intermediate host.

Laboratory diagnosis

The diagnosis of babesiosis is made from Wright- or Giemsa-stained thin blood smears. The organisms appear as small, delicate, pleomorphic ring-form trophozoites, about 1 to 2 μm in length, with a prominent chromatin dot and faintly staining cytoplasm. More mature trophozoites may appear as pyriform organisms in single, double, or the classic tetrad (Maltese cross) formation within the RBC. Fig. 28.35A shows several small, compact, ringlike trophozoites of *B. microti*; Fig. 28.35B demonstrates the irregularly arranged tetrad formation characteristic of the organism. Although they are characteristic for babesiosis, the tetrad forms are not always seen. Fig. 28.35C demonstrates a cell with a pair of trophozoites as well as cells with single organisms. The morphology initially may be confused with that of *P. falciparum*. Lack of pigment, presence of extraerythrocytic organisms, and absence of other life cycle stages serve as keys to differentiate *Babesia* from *P. falciparum*.

Patients with a normocytic hemolytic anemia may have decreased haptoglobin and elevated indirect bilirubin levels and reticulocytosis. Some patients may demonstrate elevated levels of liver enzymes and lactate dehydrogenase, and decreased numbers of platelets.

Serologic studies, such as immunofluorescence assays, can be used to detect circulating antibodies (IgM and IgG). The levels of IgM antibodies are usually elevated during the first 2 weeks and then decline. IgG titers greater than 1:1024 are generally considered indicative of recent or active infection. Although transfusion-transmitted babesiosis has been reported, there is no screening test for blood donors; obtaining a detailed donor history is the only method used to screen donors. Molecular methods to detect parasite DNA have been developed and may be used when there is a high suspicion of babesiosis but the smears remain negative.

Toxoplasma gondii

T. gondii is an obligate intracellular parasite found in mammals worldwide. Serologic studies have indicated that the rate of infection often differs among counties, but, overall, up to one third of individuals worldwide may be infected. More than 20% of the U.S. population shows serologic evidence of infection, but in parts of Europe, the proportion may be as high as 80%. Toxoplasmosis can present with a wide range of

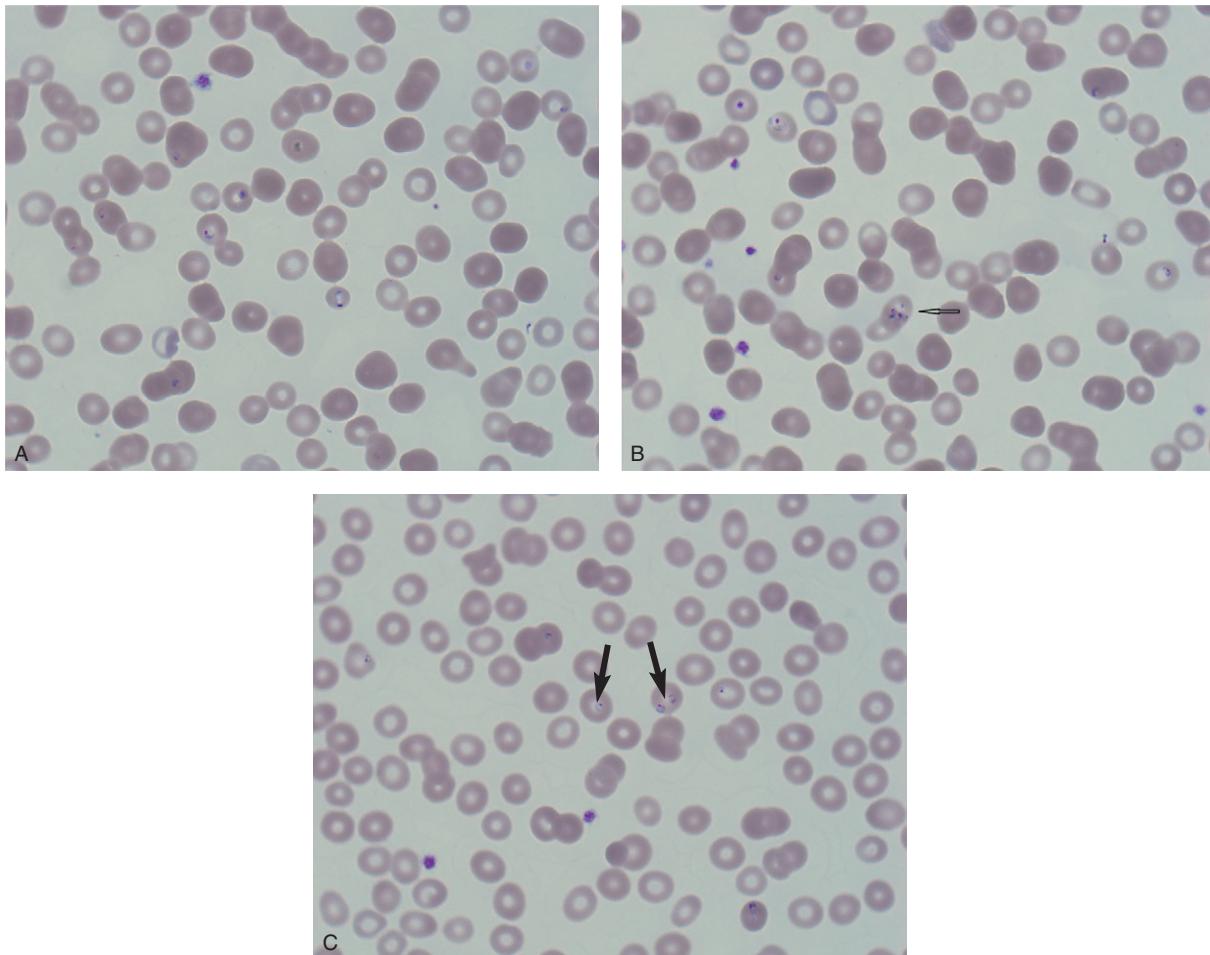


Fig. 28.35 A, *Babesia microti* trophozoites (Giemsa stain). B, *B. microti* tetrad form identified by the arrow (Giemsa stain, $\times 1000$). C, Paired trophozoites and single trophozoites identified by arrows.

clinical symptoms and complications that are often related to the immunologic status of the patient. Most immunocompetent individuals do not have serious infection, whereas HIV-positive individuals, transplant recipients, and those with defects in T cell-mediated immunity are found to have the most serious forms of infection. In addition, infants may contract the disease in utero and present with various degrees of severity.

Clinical infections

The clinical presentation of toxoplasmosis differs based on the immunologic status of the individual. Immunocompetent patients with acute infections may be asymptomatic or may have mild flulike or mononucleosis-like symptoms, including low-grade fever, lymphadenopathy, malaise, and muscle pain. Once the acute infection has resolved, the organism enters a relatively inactive stage, in which tissue cysts containing many slow-growing forms of the organism are found.

The organism may also be transmitted congenitally because of a transient parasitemia in pregnant women who have primary infection. Congenital transmission occurs when the **tachyzoites**, the motile, rapidly dividing forms in maternal circulation, cross the placenta and enter fetal circulation and tissues. Children with congenital toxoplasmosis may have a range of serious complications, including intellectual disability, microcephaly, seizures, hydrocephalus, retinochoroiditis, and blindness. If the fetus is exposed to the infection early in the pregnancy, more severe complications are likely to result. Infections acquired later in pregnancy may result in the child being asymptomatic at birth but developing complications, especially ocular problems, later in childhood.

Immunosuppressed patients, particularly those with leukemia or lymphoma and those undergoing chemotherapy, may experience a serious primary infection or reactivation of a latent infection that can manifest itself as a fulminating encephalitis and result in rapid death. Up to 10% of the deaths of patients with AIDS are caused by reactivation of latent toxoplasmosis, resulting in encephalitis. Computed tomography (CT) may demonstrate lesions in the brain that represent *Toxoplasma* spp. cysts. Pulmonary toxoplasmosis may be present in conjunction with CNS infections or can be the presenting condition; however, the organism can spread to any organ of the body.

The organism can also be transmitted via organ transplantation. In this case, the organ may contain **bradyzoites**, which are slow-growing encysted forms of the parasite responsible for chronic infections that are reactivated in the immunocompromised recipient. This frequently occurs when the organ recipient is seronegative for *T. gondii* (lacks antibodies to *T. gondii*) and the organ donor is seropositive. In these patients, the infection usually occurs within the first 3 months after transplantation and may spread to multiple organs. In the case of a seropositive transplant recipient, immunosuppressive treatment to prevent organ rejection may reactivate a latent infection in the transplant recipient.

In general, toxoplasmosis in the immunocompetent host is not treated. When treatment is necessary, a combination of antimicrobials, such as trimethoprim-sulfamethoxazole, is used for treating the infection in the tachyzoite stage. The drugs are not effective against bradyzoites in cysts located in muscle and brain tissues.

Life cycle

T. gondii has two life-cycle stages in humans: tachyzoite and bradyzoite. Tachyzoites can invade any nucleated cell and are the actively motile and reproducing forms. They are crescent shaped, are 4 to 6 μm long, and have a prominent nucleus (Fig. 28.36A). Fig. 28.36B shows lung tissue containing free tachyzoites and a number of intracellular forms. Bradyzoites are the slow-growing asexual forms found in a cystlike structure during the dormant phase of infection.

As shown in the life cycle illustrated in Fig. 28.37, humans can acquire *T. gondii* infection in several ways, such as through ingestion or inhalation of the oocyst from cat feces, soil, or water; through ingestion of undercooked meat containing the cystlike structure with bradyzoites; and through congenital transmission. Primary risk factors for acquiring the infection include cleaning a cat litter box, gardening without gloves, and eating raw or undercooked meat or unwashed vegetables and fruit. The household cat and other members of the family Felidae serve as definitive hosts for the organism. Sexual and asexual reproduction occur in the intestine of the cat, whereas only asexual reproduction occurs in humans and other intermediate hosts. The result of sexual reproduction is the production of an oocyst, which is passed in cat feces. The oocyst requires 2 to 5 days in the environment to mature and become infective.

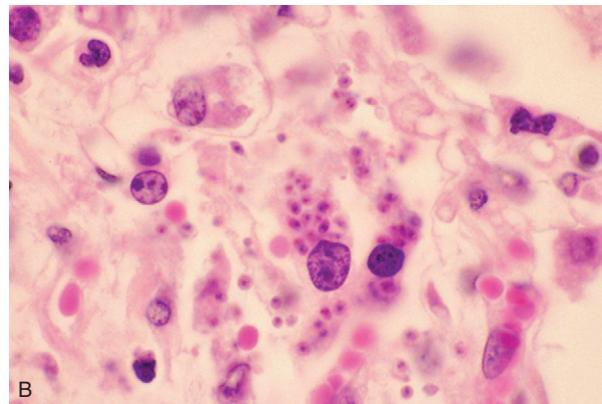
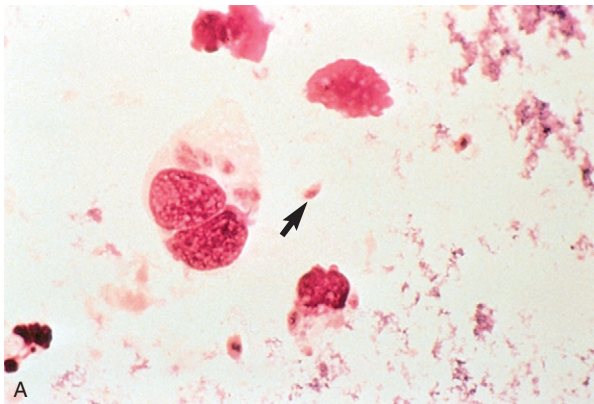


Fig. 28.36 A, *Toxoplasma gondii* tachyzoites, identified by the arrow (hematoxylin and eosin stain). B, *T. gondii* tachyzoites in lung tissue (hematoxylin and eosin stain, $\times 1000$).

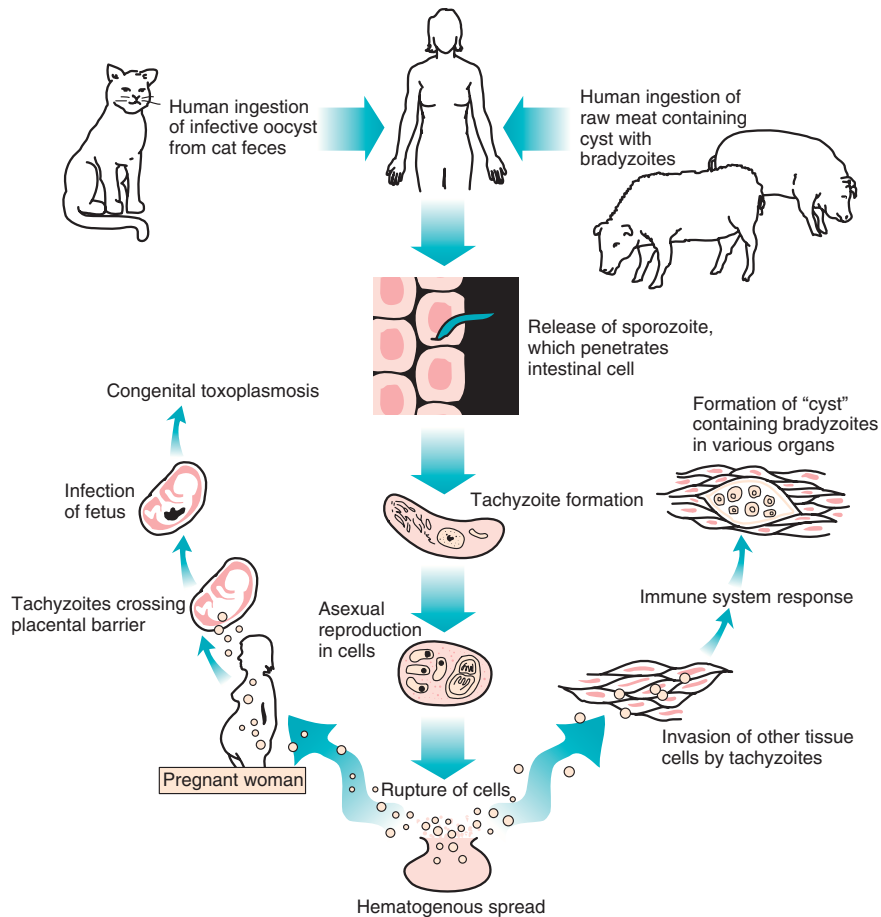


Fig. 28.37 Life cycle of *Toxoplasma gondii*.

When a human ingests the infective oocyst, changes in pH and temperature induce release of sporozoites in the oocyst into the intestine. The sporozoites penetrate the intestinal wall, gain access to the circulation, and migrate to the organs. Migration of dendritic cells infected with tachyzoites is one of several proposed methods that contribute to dissemination. These *tachyzoites*, as they are now called, are responsible for tissue destruction. They invade cells by attaching to surface proteins (using receptors, such as proteoglycans) on nucleated cells, replicate intracellularly within a parasitophorous vacuole formed by the host cell plasma membrane, cause cells to rupture, and release tachyzoites to invade other cells.

The immune system, in particular, T cells, responds strongly when tachyzoites invade the tissues. This TH1 immune response includes the production of IL-12 and IFN- γ . Natural killer (NK) cells may also play a role in control of the organism. As a result of this immune response, tachyzoites are converted to bradyzoites, and eventually the cystlike structure contains hundreds to thousands of the slowly growing and reproducing bradyzoites. At this point, the infection generally remains dormant for the life of the host unless the immune system is compromised. In the immunocompromised patient, the bradyzoites are reactivated and released from the cyst and become active tachyzoites that invade multiple organs, resulting in disseminated infection.

If a human ingests raw or undercooked meat containing the tissue cysts, the cyst wall is dissolved and bradyzoites are liberated. These organisms are resistant to digestive tract

enzymes for about 6 hours, during which time they convert to tachyzoites and invade the intestinal wall. They then gain access to the circulation. All types of meat, including lamb, pork, beef, and that of game animals, such as deer and bear, have been implicated in this mechanism.

Laboratory diagnosis

Identification of the tachyzoite or pseudocysts with bradyzoites in tissue is very difficult because no single organ is invaded. Finding free tachyzoites in CSF, bronchoalveolar lavage specimens (Figs. 28.38 and 28.39), or specimens from other sites, such as lymph nodes, is unusual unless the infection is disseminated. In disseminated toxoplasmosis, histologic stains of biopsy materials may demonstrate the cyst or, in some cases, tachyzoites. Noninvasive techniques, such as magnetic resonance imaging (MRI) and CT, may be used in the diagnosis for patients suspected of having disseminated toxoplasmosis.

The levels of antibodies to the organism show a rapid increase during infection, and tests for antibodies are most commonly used for diagnosis. Indirect fluorescent antibody tests and EIAs with *T. gondii* organisms as the antigen are routinely used for diagnosis. Because most patients have an antibody titer to the organism, interpretation of the titer must be linked to clinical symptoms and the patient's history. A significant rise in titer (fourfold) between acute-phase and convalescent specimens may indicate acute infection. An IgM-specific test may also be used to diagnose acute infections and can be useful in the diagnosis of primary toxoplasmosis

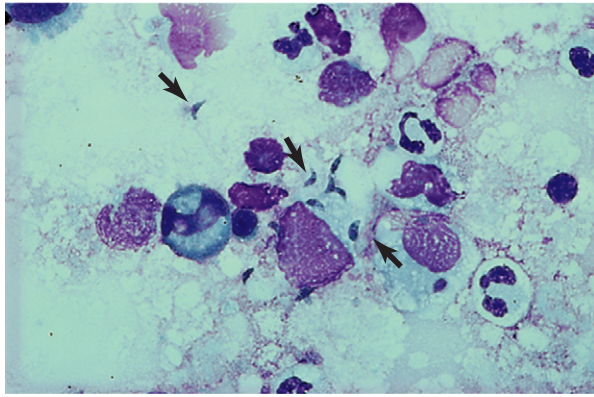


Fig. 28.38 Bronchoalveolar lavage cytocentrifuge preparation, Wright-Giemsa stain, Crescent-shaped cells with central nucleus (arrows). Morphology consistent with trophozoites (tachyzoites) of *Toxoplasma gondii* (see Fig. 28.39).

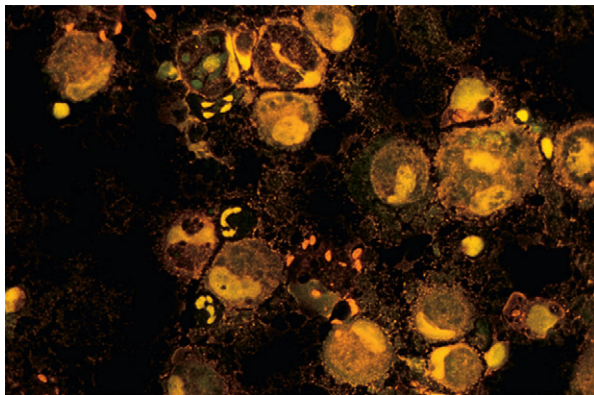


Fig. 28.39 Bronchoalveolar lavage. Cytocentrifuge preparation, acridine orange stain, fluorescence microscopy, Crescent-shaped cells composed of RNA. Morphology consistent with trophozoites (tachyzoites) of *Toxoplasma gondii*.

in pregnant women suspected of having been exposed to the organism or in neonates in whom congenital infection is suspected. IgM titers usually peak within the first month of infection. Antibody titers may be unreliable in immunocompromised patients because these patients lack the ability to produce sufficient antibody to cause a significant increase in titer. PCR assays to detect *T. gondii* DNA may be used for CSF or amniotic fluid and are useful in detecting congenital infection in utero. Molecular methods are also used in bone marrow transplant recipients to detect infection.

Opportunistic intestinal Apicomplexa species

Cryptosporidium spp. are recognized for causing self-limiting GI infection in immunocompetent hosts. However, they have also been identified as opportunistic pathogens causing severe infection in immunocompromised hosts, particularly in those with AIDS. *Cryptosporidium* spp. are obligate intracellular parasites transmitted by ingestion of the infective oocyst. These organisms are transmitted by the fecal-oral route—ingestion of contaminated water or food or, for some, by direct contact with fecal material containing oocysts. The oocysts are infective at a low dose (as few as 10 oocysts) and are resistant to common chlorine- and ammonia-based disinfectants. Most infections are reported in summer, when there is increased recreational water activity. Foodborne infections

have been associated with ingestion of raw foods or unpasteurized drinks.

Sexual and asexual reproduction occur in the human intestinal tract. Over 30 *Cryptosporidium* species are recognized; *Cryptosporidium* was initially identified as causing zoonotic infections in veterinarians and other animal handlers. The parasites can infect a range of hosts including mammals, birds, and reptiles. Cows are an important reservoir for human disease. Now, over 20 species have been found in humans; the most common are *Cryptosporidium hominis* and *Cryptosporidium parvum*. Cryptosporidiosis is the leading cause of waterborne disease outbreaks in the United States. Outbreaks in daycare centers and from environmental contamination of municipal water supplies have been reported. In the 1980s, cryptosporidiosis was identified as a major opportunistic infection in patients with AIDS and classified as an AIDS-defining illness. In a 2019 report, the CDC estimated that 823,000 cases of cryptosporidiosis occur annually.

Clinical infections

In immunocompetent patients, the organisms cause a transient profuse, watery diarrhea along with mild to severe nausea and vomiting, headache, and cramps. The mucosa is inflamed, and there is an influx of segmented neutrophils, macrophages, and lymphocytes. The onset is rapid (within 3 to 7 days after ingestion of the oocyst), but the infection is self-limiting. Symptoms resolve in several weeks, although infections can last up to 1 month. Infection begins in the small intestine but may spread to the large intestine. In endemic areas, the first infection occurs in early childhood and is symptomatic with profuse, watery diarrhea, but subsequent infections are mild to asymptomatic. Antibodies (IgG, IgA, and IgM) are produced, but it is not clear how much of a protective role they play. Antibodies developed against one of the organisms can provide protection against infection by the same species, but antibodies developed against one species may provide only incomplete protection against another species. In addition, the host mounts a cell-mediated immune response (T cells and NK cells) that includes production of several cytokines, including IFN- γ and IL-12.

The organism alters osmotic pressure in the gut, with a resulting influx of fluid. The diarrhea is cholera-like, with bits of mucus and little fecal material. Fluid loss has been reported to range from 3 to 6 L/day to as much as 17 L/day. In addition to weight loss, patients show signs of dehydration and electrolyte imbalance. In patients with AIDS whose cell-mediated immunity is compromised and whose CD4 count is less than 50 cells/ μ L, the infection is more likely to become fulminant or life-threatening or spread to extraintestinal sites.

In chronic cases, the intestinal villi may show signs of atrophy caused by inflammation, and the brush border of the cells is disrupted because of invasion by the organism. This damage alters intestinal permeability and can result in decreased uptake of fluids, electrolytes, and nutrients. Malabsorption syndrome may occur. No antimicrobial is completely effective against this infection. Paromomycin and azithromycin, which can suppress the infection, have been used, with mixed results. Nitazoxanide, a relatively new agent, has been used for treating *Giardia* and *Cryptosporidium* infections in immunocompetent children and adults. It shortens the duration of diarrheal episodes and appears to be the only one of the drugs tested that has even partial effectiveness in immunocompromised individuals.

Life cycle

The sexual and asexual life cycles of *Cryptosporidium* occur in the same host. Life cycle stages, as shown in Fig. 28.40, develop under the brush border of the intestinal mucosal epithelial cells. Oocysts are infective when passed and may be ingested in contaminated water or food or passed by person-to-person contact. Ingestion of the infective oocyst initiates the asexual cycle (sporogony) with the release of sporozoites. Sporozoites attach to receptors on the intestinal mucosal border, penetrate an intestinal epithelial cell and create a parasitophorous vacuole between the cell membrane and cytoplasm, and mature into trophozoites in this intracellular but extracytoplasmic location. Once trophozoites have matured, the development of meronts with merozoites begins. The nucleus and cytoplasm divide to form individual organisms known as *merozoites*. When the meront ruptures, merozoites are released and penetrate other cells to continue asexual reproduction or to transform

into a microgamete or macrogamete. Fertilization of the macrogamete results in formation of the oocyst, which contains four sporozoites. Two types of oocysts may be formed, the thin-walled oocyst, which ruptures within the intestine and results in autoinfection, and the thick-walled, fully sporulated oocyst, which is infective when passed in feces. Key factors in the life cycle of this organism that contribute to its pathogenicity include the following:

- The oocysts are infective when passed in feces.
- Rupture of thin-walled oocysts in the intestine creates the potential for continual autoinfection.
- Patients may remain infective and continue to shed oocysts for a time after the diarrhea ceases.

Laboratory diagnosis

The small size (4 to 6 μm), refractile appearance, and round shape of the oocyst make detection difficult with routine

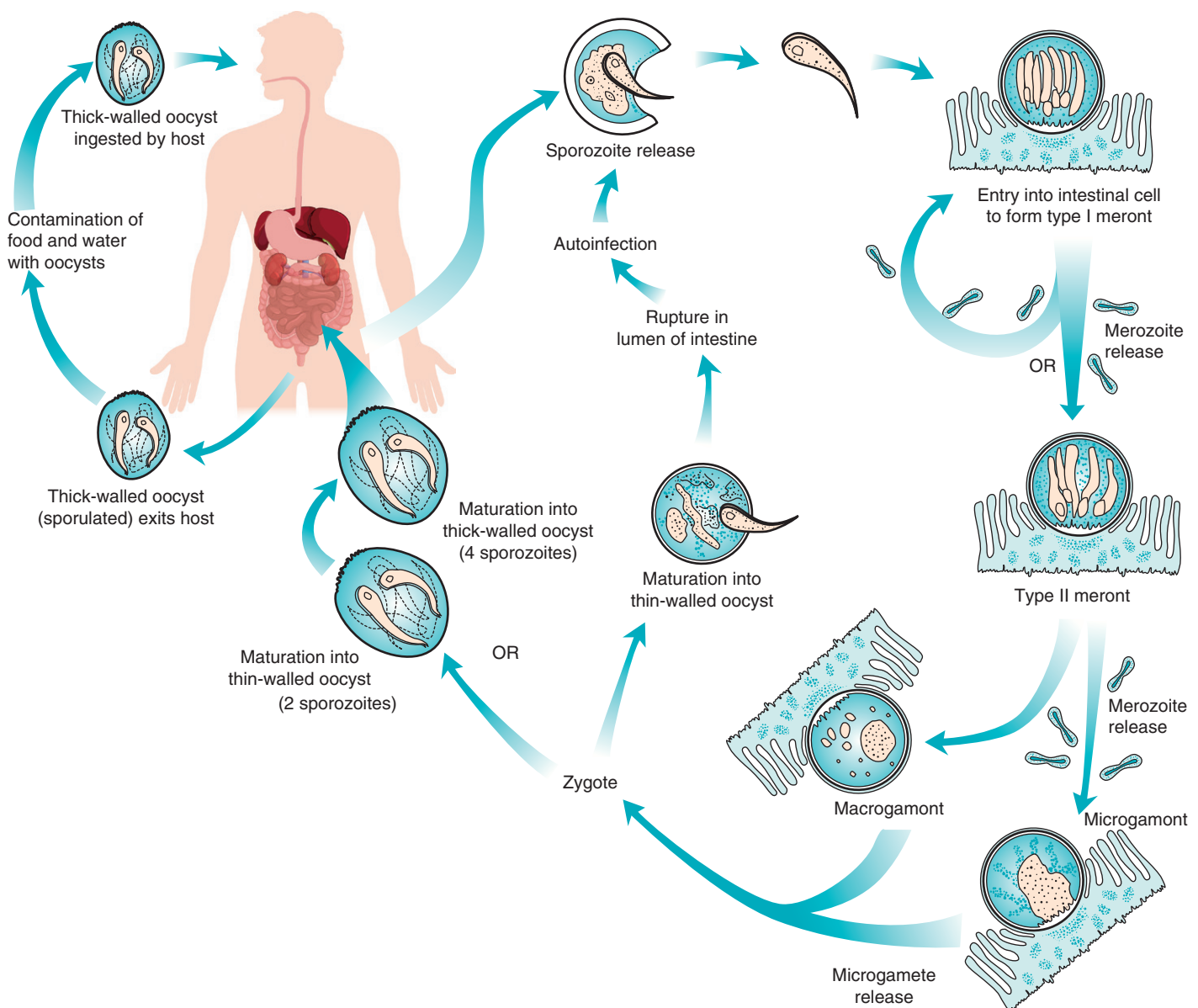


Fig. 28.40 Life cycle of *Cryptosporidium* sp.

concentration procedures because the organism may resemble a yeast or an RBC. More oocysts are seen in liquid stool than in formed stool. Trichrome and iron hematoxylin stains are not useful permanent stains for identification of *Cryptosporidium* spp. The recommended detection methods for *Cryptosporidium* infection are the modified acid-fast stain, an antigen detection test, and a DFA test using a monoclonal antibody directed against *Cryptosporidium* spp. These methods do not distinguish the species. With the acid-fast stain, the organism appears as a bright red sphere, which distinguishes it from yeasts, which stain green. Figs. 28.41, 28.42, and 28.43 show *Cryptosporidium* oocysts stained with modified acid-fast stain.

Studies indicate that antigen detection by monoclonal antibody tests demonstrates greater sensitivity and specificity than the modified acid-fast stain. EIA methods can also be used but are less sensitive (sensitivity of 70%) than fluorescent antibody methods. More recently, lateral-flow immunochromatographic assays have become available. These assays are highly specific (>99%), but the sensitivity ranges from 68% to 100%. Antigen-detection methods have become popular in clinical laboratories for their ease of use—they do not require an experienced microscopist.

Cystoisospora belli

Cystoisospora belli (formerly *Isospora belli*) is an opportunistic organism seen less frequently than *Cryptosporidium* spp. It is

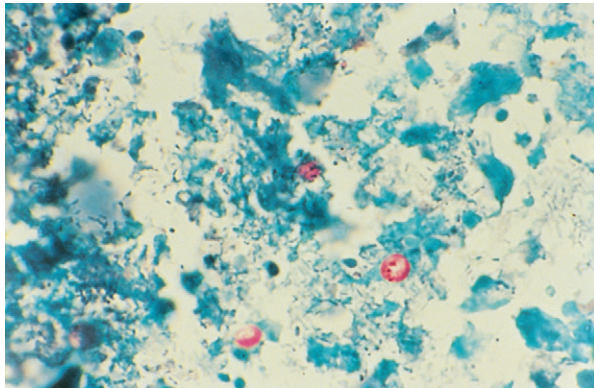


Fig. 28.41 *Cryptosporidium* oocysts (modified acid-fast stain, $\times 1000$).

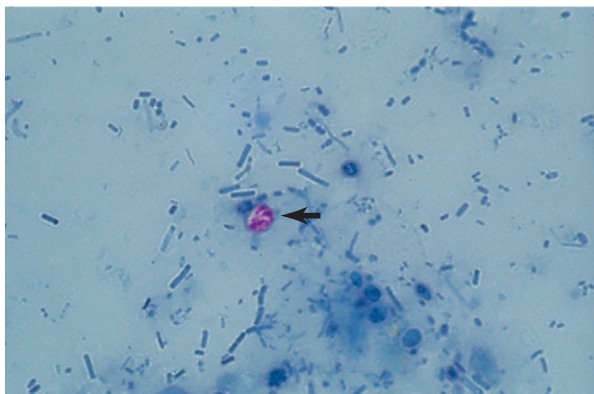


Fig. 28.42 Watery, frothy diarrheic stool, smear, acid-fast stain, ($\times 1000$). Acid-fast oocysts (4 to 6 μm). Sporulated oocysts containing four sporozoites (arrow). Morphology consistent with *Cryptosporidium* sp.

known to infect only humans. Most patients infected with *C. belli* are asymptomatic, but symptoms, such as low-grade fever, headache, diarrhea, and colicky abdominal pain, may be present. Acute infections with *C. belli* are usually clinically indistinguishable from those with *Cryptosporidium* spp. The infection is self-limiting and usually resolves in several weeks in an immunocompetent host. The infection is often more serious in immunocompromised patients, including those with AIDS and those with conditions such as Hodgkin disease, lymphoproliferative disorders, or lymphoblastic leukemia. These patients may have watery diarrhea and concurrent weight loss, or chronic infection may develop. Treatment with trimethoprim-sulfamethoxazole has been effective in eliminating diarrhea, but patients often show recurrence of infection when therapy is discontinued.

The life cycle of *C. belli* is similar to that of *Cryptosporidium* spp. in that it requires only one host but occurs within the cytoplasm of epithelial cells of the small intestine. The oocyst of this organism is not infective when passed in the feces and requires 24 to 48 hours outside the body to become infective. There is no thin-walled oocyst to cause continued autoinfection. The mature oocyst of *C. belli* is oval, 20 to 33 μm by 10 to 19 μm , with a hyaline cell wall. The immature oocyst usually shows a single **sporoblast** (an early stage in the development of a sporocyst, before differentiation of the sporozoites). The mature oocyst has two **sporocysts**, which are walled bodies. Each sporocyst contains four elongated sporozoites. Oocysts can be seen in wet mounts.

The modified acid-fast stain has been used to detect *C. belli*. The oocyst wall does not stain but often shows a faint outline because of precipitated stain, and the sporoblasts or sporocysts stain dark red. Like oocysts of *Cyclospora cayetanensis*, the oocysts of *C. belli* will autofluoresce a bluish color at 330 to 365 nm and a bright green at 450 to 490 nm. The size and shape of the oocyst serve as the identification characteristics. Fig. 28.44 shows the characteristic appearance of an acid-fast stain of the oocyst of *C. belli*.

Cyclospora cayetanensis

C. cayetanensis is a foodborne and waterborne organism causing endemic and epidemic diarrheal disease. Humans are the only known host for this organism, but other *Cyclospora* spp. are found in animals. The organism was first linked to human

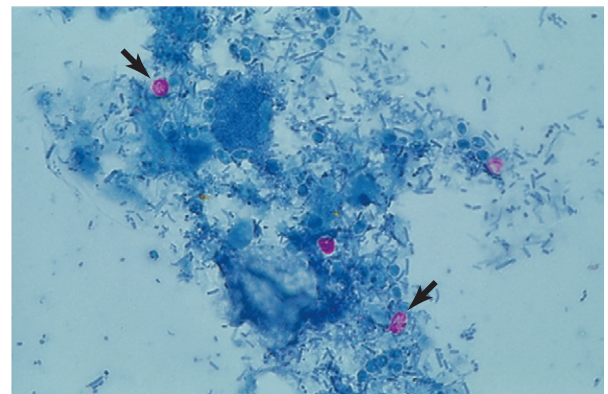


Fig. 28.43 Watery, frothy diarrheic stool, smear, acid-fast stain. Acid-fast oocysts (4 to 6 μm) (arrows) ($\times 1000$). The measurement is taken to differentiate this oocyst from the 8- to 10- μm oocysts of *Cyclospora* spp. (see Fig. 28.45B).

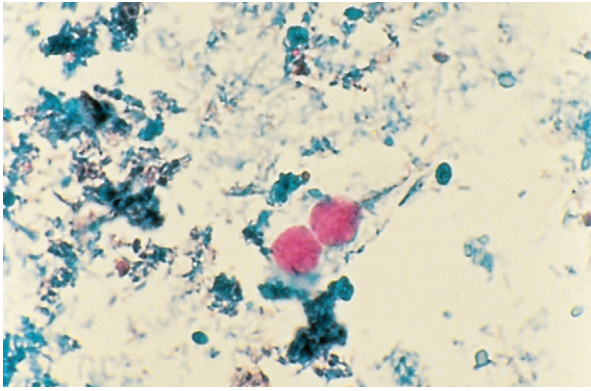


Fig. 28.44 *Cycloispora belli* oocysts (modified acid-fast stain, $\times 1000$).

disease in the 1990s, when it was found in stool specimens. It was originally thought to be a *Cyanobacterium*-like body, blue-green alga, or large *C. parvum*.

C. cayetanensis is endemic in Nepal, Peru, Guatemala, and Haiti, but outbreaks have been reported in many countries, including those in Central and South America, parts of the Caribbean, India, and Europe. The usual mode of transmission is ingestion of fecally contaminated water or food. Most outbreaks in endemic areas occur during the rainy season. Persons living in these areas may acquire some level of immunity that increases resistance to future infection. Individuals who travel to endemic areas are susceptible to traveler's diarrhea caused by *C. cayetanensis*. The organism has also been linked to several large foodborne outbreaks in North America. Most of the outbreaks were associated with the consumption of contaminated imported fresh produce, especially raspberries and strawberries from endemic areas of Central America. Subsequently, there have been isolated outbreaks associated with imported vegetables, such as mesclun lettuce, basil, and snow peas. The infective dose is not known, but it is thought to be relatively low, between 10 and 100 oocysts.

Although the organism is most commonly encountered in immunocompetent patients, it may be considered an opportunistic infection in patients with AIDS. Symptoms of infection with *C. cayetanensis* differ based on age, infective dose, and host immune status. The incubation period is approximately 1 week. The organism infects cells in the upper portion of the small intestine and causes frequent, watery, but nonbloody stools that may alternate with bouts of constipation. Symptoms, which can mimic those of cryptosporidiosis or cystoisosporiasis, include anorexia, fatigue, weight loss, abdominal cramping and bloating, vomiting, low-grade fever, and nausea. Patients often report a flulike syndrome before the onset of diarrhea. In immunocompetent hosts, the symptoms are self-limiting and persist for several weeks but may occur in a relapsing pattern for up to 2 months.

Immunocompromised patients have prolonged course and can be symptomatic for as long as 4 months. Some cases of malabsorption caused by inflammation and damage to the intestinal villi and a few cases of extraintestinal infection have occurred in immunocompromised patients. In endemic areas, infections are common in children 2 to 10 years of age, and most infections resolve spontaneously. Trimethoprim-sulfamethoxazole is used to treat the infection; when treatment is started, symptoms usually abate within several days.

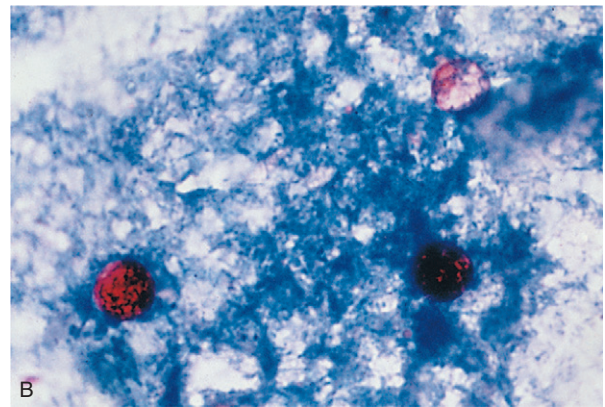
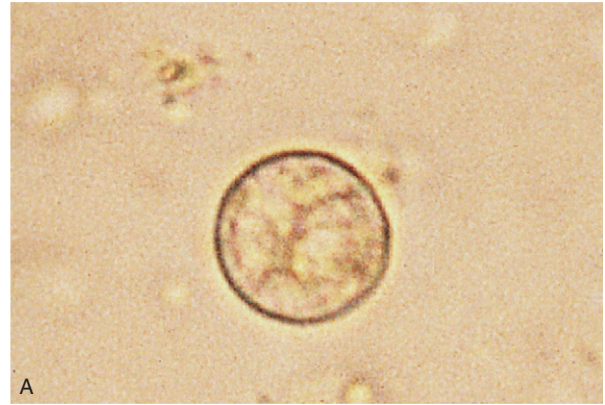


Fig. 28.45 *Cyclospora cayetanensis* oocyst. **A**, Wet mount. **B**, Modified acid-fast stain. (**A**, original magnification $\times 1000$; **B**, $\times 1000$.)

The organism shares the general life cycle characteristics of other intestinal Apicomplexa species. Once the mature oocyst has been ingested, the presence of bile and trypsin in the small intestine helps trigger release of sporozoites, which invade the intestinal cells. Two types of meronts develop: type I, which produce merozoites that infect other intestinal cells (asexual cycle), and type II, which progress to the sexual stage. Once fertilization occurs, a zygote forms and develops into an immature oocyst that is passed in feces. Unlike *Cryptosporidium*, however, immediate person-to-person transmission is unusual because these oocysts require about 1 to 2 weeks outside the body to mature and become infective.

The oocyst is similar to that of human *Cryptosporidium* spp. but larger, with an average size of 8 to 10 μm . In wet mounts, the oocyst appears nonrefractile, spherical, and unsporulated, with multiple internal globules or granules. Sporulation occurs after several days, resulting in the production of two sporocysts with two sporozoites each. The organism does not stain with traditional trichrome or iron hematoxylin stains. The modified acid-fast stain demonstrates variably staining organisms, from dark pink to almost colorless (ghost forms), with no visible internal structures. Fig. 28.45A shows an oocyst of *C. cayetanensis* in a wet mount, and Fig. 28.45B shows an oocyst in an acid-fast stain. For comparison, Fig. 28.43 shows acid-fast *Cryptosporidium* oocysts that measure 4 to 6 μm (arrows).

One distinguishing characteristic of *C. cayetanensis* is autofluorescence, exhibiting a bright blue fluorescence (330 to 365 nm) or mint green fluorescence (450 to 490 nm) under ultraviolet light. Nucleic acid detection methods developed in reference and research laboratories are often used for

identification during outbreaks. Serologic methods to detect antibodies and antigen detection assays are not available.

Microsporidia

In the mid-1980s, a group of organisms were linked to infections in patients with HIV infection. These organisms, collectively referred to as *microsporidia*, are obligate intracellular, parasites common to invertebrates and other animals. Originally considered protists, they have been reclassified as fungi based on chitin present in the spore wall and rRNA sequencing. There are more than 200 genera and 1500 species identified, with many being reclassified based on molecular sequencing. At least nine genera (*Anncaliia*, *Encephalitozoon*, *Endoreticulatus*, *Enterocytozoon*, *Nosema*, *Pleistophora*, *Trachipleistophora*, *Tubulinosema*, *Vittaforma*, and the unclassified collective group *Microsporidium*) have been implicated in human infections. Although infection with the organisms is often linked to HIV infection, microsporidia have been identified in organ transplant recipients, older adults, and patients with traveler's diarrhea. The organisms are capable of infecting a wide variety of human organs, including intestine, eyes, muscles, liver, kidneys, and CNS.

Clinical infections

Symptoms differ with the species and organ infected. Intestinal infections are most often caused by *Enterocytozoon bieneusi* or *Encephalitozoon intestinalis*, and infection in immunocompetent hosts is characterized by diarrhea, cramps, loss of appetite, and fatigue. Dehydration and weight loss are sometimes seen. Infected patients have four to eight liquid or loose stools a day, and symptoms may persist for up to 8 months, with spontaneous exacerbations and remissions. In patients with AIDS, infection is chronic, and the diarrhea may persist for several years, with malabsorption of fats and vitamin B₁₂ and cachexia as additional complications. Risk factors in patients with AIDS include sexual transmission, a CD4 cell count of less than 100/ μ L, exposure to water during swimming, and contact with animals. Dissemination of *E. intestinalis*, especially to the urinary tract, gallbladder, or respiratory tract, has been seen. Dissemination to muscles may result in weakness and pain. Patients with AIDS can develop encephalitis, nephritis, or keratoconjunctivitis. Contact lens wear is a risk factor for corneal infection. Albendazole has been used to treat infections with *Encephalitozoon* spp., but most other microsporidial organisms are resistant to this and other drugs. Metronidazole may provide some relief, but the symptoms recur when use of the drug is discontinued. Disseminated infections have also been reported.

Life cycle

The infective stage for humans is the environmentally resistant spore, which can survive outside the host for as long as 1 year. Once the infective spore is ingested, shifts in pH and ionic concentration in the intestinal tract trigger infection. The organism gains access to host cells by emergence of a polar tube through the spore wall and its insertion through the cell membrane. This insertion is thought to be mediated by a series of molecular interactions between the tip of the

polar tube and receptors on the cell membrane. The contents of the spore (sporoplasm containing a nucleus, ribosomes, Golgi apparatus) are then transferred into the cell. Within the host cell, the sporoplasm divides and develops into meronts. These structures subsequently develop into sporoblasts that differentiate into sporonts. As these structures mature into spores, they develop a thick membrane and the polar tube. The host cell ruptures, and spores are released to penetrate other host cells to repeat the reproductive cycle or be passed from the body. In the case of intestinal infection, spores are passed in feces; in infection of the urinary tract, they can be detected in urine.

Laboratory diagnosis

Microsporidia can be found in stool, bronchoalveolar lavage fluid, sputum, CSF, conjunctival swabs, corneal scrapings, and other specimens. Initially, identification methods were limited to finding the small spores in Giemsa-stained tissue sections or in electron microscopic examination of biopsy specimens. Electron microscopy must be used to identify the species of the organism. Speciation is based on the number of coils in the polar tubule, septations in the spore, and size.

Routine O & P examination will not detect the spores in a fecal specimen. However, staining of formalin-preserved feces using the Weber modification of the trichrome stain and the Ryan trichrome blue stain can be used to detect microsporidial spores. The small size of the spores, however, makes them easy to overlook in clinical specimens. A thin smear of feces must be used so debris does not obscure the small, faintly staining spores. Fig. 28.46 shows the small spores (1.5 to 4.0 μ m) in a chromotrope stain of a fecal specimen. Spores stain pink to red and may have a diagonal or equatorial band that helps distinguish them from bacteria or yeasts. Background staining in the Weber stain is pale green (Fig. 28.47), whereas in the Ryan stain it is blue. A Gram-chromotrope stain will produce spores that are dark violet and demonstrate an equatorial band.

Calcofluor white, a fluorescent stain used for the detection of fungi, can also be used to screen specimens for microsporidial spores. The stain is a chemofluorescent agent, such as the Remel Calcofluor White Stain Kit (Thermo Fisher Scientific, Waltham, MA) or Fungi-Fluor (Polysciences, Warrington, PA).

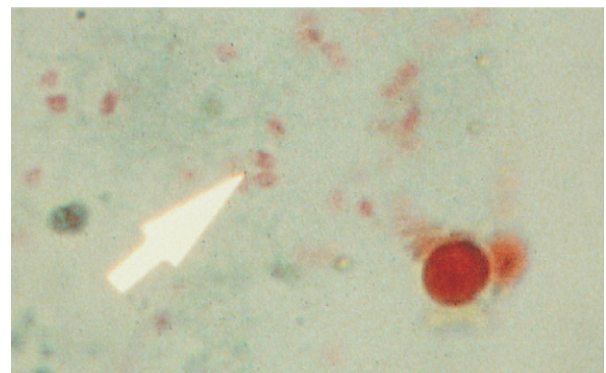


Fig. 28.46 Microsporidia spores identified by the arrow (chromotrope stain, $\times 1000$). (Courtesy Texas Department of State Health, Austin, TX.)

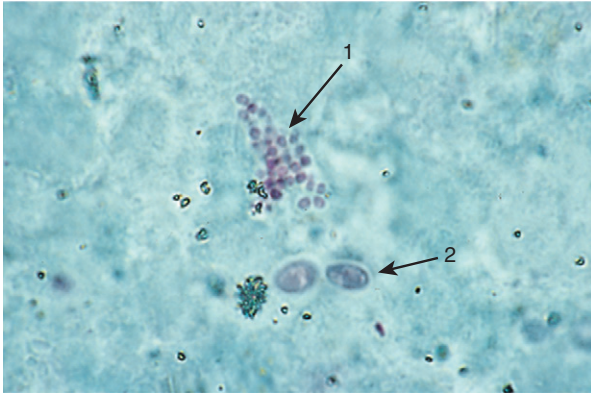


Fig. 28.47 Diarrheic stool, smear, modified Weber stain, light microscopy, $\times 2000$ oil. Parasite spores, small ($1.5 \times 0.9 \mu\text{m}$). Morphology consistent with *Enterocytozoon* sp. (arrow 1) (see Fig. 28.48). Compare with size of the yeast (arrow 2).

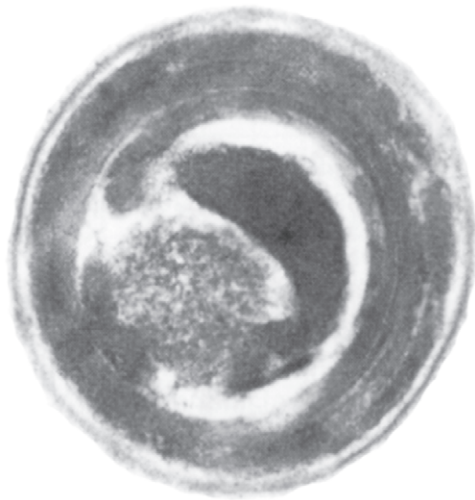


Fig. 28.48 Diarrheic stool from concentration, transmission electron microscopy, $\times 57,000$. Endospore layer and polar tubes are present. Morphology is consistent with *Enterocytozoon bieneusi*.

The stain is nonspecific and binds to chitin in the spore wall. Therefore the presence of spores should be confirmed by using one of the modified trichrome stains. Species-specific indirect fluorescent antibody staining is useful to identify spores in fluids, such as urine, or in biopsy specimens. Species-specific PCR assays are also available for identification. The transmission electron microscope micrograph in Fig. 28.48 shows an endospore layer and polar tubes. The morphology is consistent with *Enterocytozoon bieneusi*.

Helminths

Helminth infections in humans are caused by flukes, tapeworms, or roundworms. Humans become infected by directly ingesting eggs (ova), by ingesting larvae in an intermediate host, or through direct larval penetration of the skin. Adult forms do not multiply in the human body; therefore the number of adult worms present is related to the number of eggs or larvae ingested. The pathologic consequences and severity of infection are related to the number of adults

present, commonly referred to as the *worm burden*. Patients with only a few adult worms are usually asymptomatic, whereas a patient with a large number of adults often shows clinical symptoms. Most of the parasites inhabit the intestinal tract, but species also infect the liver, lungs, lymphatics, and blood vessels.

Flukes

Flukes (trematodes) are members of the phylum Platyhelminthes, or flatworms. Most infections are seen in people from East Asia, Africa, South America, and some areas of the Caribbean. Adults can range in size from a few millimeters to almost 8 cm. With the exception of the blood flukes, adult flukes are dorsoventrally flattened and have an oral sucker at the anterior end and a ventral sucker located midline, posterior to the anterior sucker. Except for the blood flukes, flukes are hermaphroditic, possessing male and female reproductive organs.

Fig. 28.49 shows the generalized life cycle of the liver, lung, and intestinal flukes. In all species, eggs must reach water to mature, and all have a snail species as the first intermediate host. The **miracidium** (first-stage larva) is ingested by a snail while within the egg or is released from eggs and penetrates the snail. Within the snail, a complex development of germinal tissue occurs, resulting first in sporocysts, which contain undifferentiated germinal structures, and then in rediae, which contain partially differentiated germinal material. The **cercaria** (second-stage larva) develops within the redia and is released into the water. Then, depending on the species, the cercaria attaches to aquatic vegetation or invades the flesh of aquatic organisms. At this stage, the organism is referred to as a **metacercaria** and is infective for humans. Except for the schistosomes, which infect humans by direct cercarial penetration, infection occurs when an individual ingests the metacercaria in raw or undercooked aquatic animals or on water vegetation. Prevention includes adequate cooking of water vegetation, fish, and crustaceans. In the case of the blood flukes, individuals should wear clothing and shoes to prevent cercarial penetration. Praziquantel is the recommended treatment for intestinal flukes, the liver fluke *Clonorchis sinensis*, the lung fluke *Paragonimus westermani*, and blood flukes. The liver fluke *Fasciola hepatica* is treated with triclabendazole.

The egg is the primary diagnostic stage. It is best detected on a wet mount of a concentrated specimen. Routine concentration procedures for feces, such as FES, may be used. The zinc sulfate method is not satisfactory, however, because all eggs except those of schistosomes are operculated. With the zinc sulfate method, the operculum might open and release the contents or cause the egg to sink. Table 28.7 compares the characteristics of fluke eggs.

Intestinal flukes

Fasciolopsis buski, known as the *giant intestinal fluke*, is found in the Far East, including China, Vietnam, and India. Dogs and pigs serve as reservoir hosts. Humans acquire the infection by ingesting metacercariae on freshwater vegetation, such as bamboo shoots and water chestnuts. Adults of *F. buski* live in the duodenum, where they cause mechanical and toxic damage. Inflammation and ulceration of the mucosa

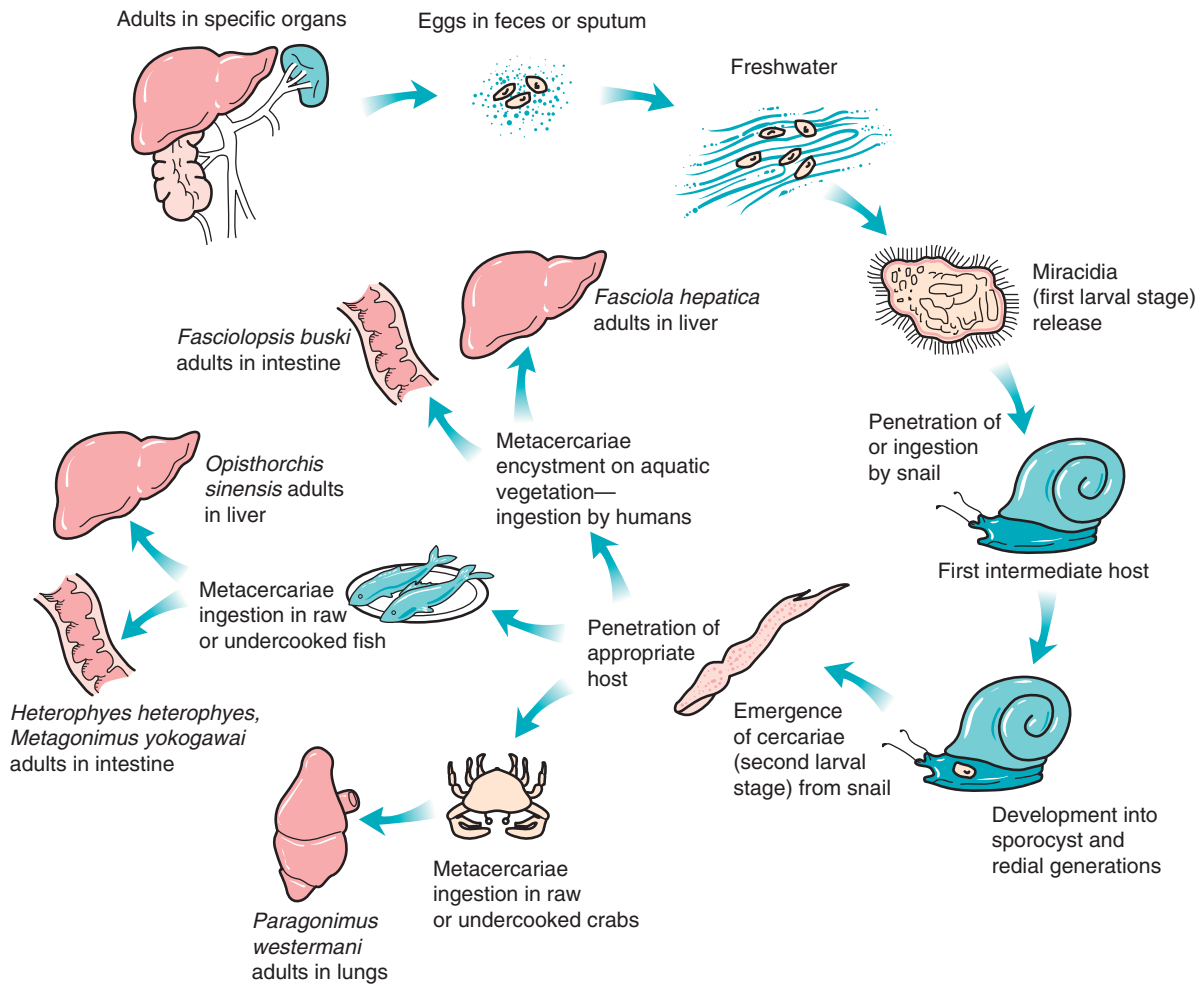


Fig. 28.49 Life cycle of liver, lung, and intestinal flukes.

may be present. Heavy infections result in persistent diarrhea, anorexia, edema, ascites, nausea and vomiting, and/or intestinal obstruction.

Finding the adult or egg is diagnostic, although the egg is more commonly seen. The adult is flattened, is 2 to 7 cm long, and lacks the cephalic cone seen in *Fasciola hepatica*. Adults are usually not seen in a stool sample unless it is a purged specimen. Eggs are yellow to brown, average 130 to 140 μm by 80 to 85 μm in size, and have a small, relatively inconspicuous operculum. They are unembryonated when passed (Fig. 28.50). These eggs are identical to those of *F. hepatica* and, when seen, should be reported as *Fasciolopsis buski*/*Fasciola hepatica* eggs.

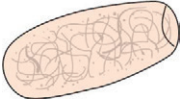
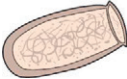
Metagonimus yokogawai and *Heterophyes heterophyes* are two small flukes found in the Far East and Middle East. Humans acquire infection with these organisms by ingesting the metacercariae in undercooked or raw fish. Adults live in the small intestine and produce few symptoms. A patient with a heavy worm burden may have diarrhea, colic, and loose stools, with a large amount of mucus. Adults of both species are small (1 to 2 mm) and delicate. Eggs serve as the primary diagnostic stage. They are 28 to 30 μm long and have a vase or flask shape. They are embryonated and operculated, with inconspicuous shoulders at the operculum. Eggs of these species resemble each other and those of *Clonorchis sinensis*.

Liver flukes

Fasciola hepatica, the sheep liver fluke, is seen in the major sheep-raising areas of the world, including parts of the southwestern United States. In addition, the organism is found in some cattle-raising areas. In sheep, the organism causes a disease known as *liver rot*, which is characterized by liver destruction. Humans acquire the infection by ingesting metacercariae on raw water vegetation, especially watercress. The larvae reach the liver by migrating through the intestinal wall and peritoneal cavity. Adults live in the biliary passages and gallbladder and rarely cause overt symptoms because infections are light. Tissue damage during migration through the liver can result in eosinophilia, an inflammatory reaction, secondary bacterial infection, and fibrosis in the biliary ducts. Heavy infections induce diarrhea, upper right quadrant abdominal pain, hepatomegaly, cirrhosis, and liver obstruction, with resulting jaundice. Chronic infections are usually asymptomatic.

Adults are approximately 3 cm long and have a prominent cephalic cone. The unembryonated, operculated eggs are carried in the bile to the intestinal tract and are passed in the feces. The size is 130 to 150 μm $\times$ 60 to 90 μm . They are almost indistinguishable from eggs of *F. buski* and are reported as *F. buski*/*F. hepatica* eggs (see Fig. 28.50).

Table 28.7 Comparisons of fluke eggs

Organism	Average size (μm) and shape	Other identifying features
<i>Fasciola hepatica</i> 	130 $\times$ 60–90, ellipsoidal	Small, indistinct operculum Yellow-to-brown color Unembryonated when passed
<i>Fasciolopsis buski</i> 	130 $\times$ 80–85, ellipsoidal	Cannot be distinguished from <i>F. hepatica</i> Unembryonated when passed
<i>Paragonimus westermani</i> 	80–115 $\times$ 48–60, oval	Brown, thick shell Slightly flattened operculum Shoulders at operculum Unembryonated when passed
<i>Clonorchis sinensis</i> 	29–35 $\times$ 12–19, vase shaped	Domed operculum Prominent shoulders Knob at end opposite operculum Embryonated when passed
<i>Heterophyes heterophyes</i> and <i>Metagonimus yokogawai</i> ^a	28–30 $\times$ 15–17, vase shaped	Operculated Shoulders not distinct Small knob Embryonated Similar to <i>C. sinensis</i>
<i>Schistosoma mansoni</i> 	115–175 $\times$ 45–75, oval	Lateral spine No operculum Embryonated when passed
<i>Schistosoma haematobium</i> 	110–170 $\times$ 40–70, oval	Rounded anterior Terminal spine Embryonated when passed
<i>Schistosoma japonicum</i> 	60–95 $\times$ 40–60, round to slightly oval	Small, inconspicuous, hooked lateral spine Embryonated when passed

^aThe eggs of these two species, *C. sinensis*, and *Opisthorchis*, are almost indistinguishable.

The Chinese or Oriental liver fluke, *Clonorchis sinensis*, is geographically limited to the Far East, where dogs and cats serve as reservoir hosts. *Opisthorchis* spp. are additional liver flukes found in humans. *Opisthorchis viverrini* is known as the Southeast Asian liver fluke, and *Opisthorchis felinus* is known as the cat liver fluke. The adults live in the distal portion of the bile ducts. As with *F. hepatica*, light infections produce few or no symptoms. Repeated or heavy infections cause inflammation because of mechanical irritation, fever, diarrhea, pain, fibrotic changes, or obstruction of the bile duct. Humans acquire the infection by ingesting the metacercariae in raw, undercooked, or pickled fresh-water fish.

The diagnosis is made by finding ova in feces or, occasionally, in duodenal aspirates. Adults are thin, tapered at both ends, and 1 to 2.5 cm long. It is difficult to

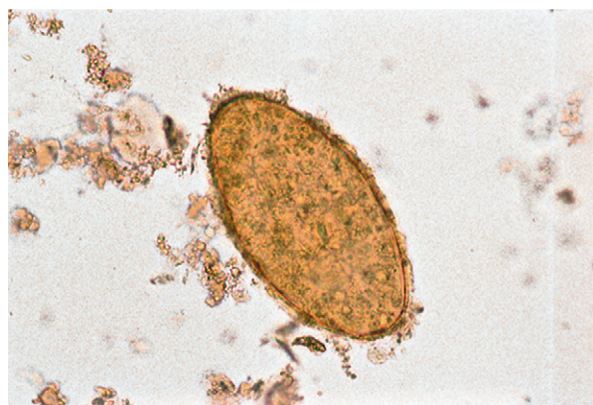


Fig. 28.50 *Fasciolopsis buski*/*Fasciola hepatica* egg (unstained, $\times 400$).



Fig. 28.51 *Clonorchis sinensis* egg (unstained, $\times 1000$).

distinguish the ova of *Clonorchis*, *Opisthorchis*, *Heterophyes*, and *Metagonimus*. The egg is 29 to 35 μm long, embryonated when passed, flask shaped, and operculated, with prominent shoulders at the operculum and a knob at the opposite end (Fig. 28.51).

Lung flukes

Organisms of the genus *Paragonimus* usually infect tigers, leopards, dogs, and foxes. About 30 species of *Paragonimus* have been named, and 9 have been identified as human parasites. An estimated 20 million people suffer from paragonimiasis. *Paragonimus westermani*, the lung fluke, is found primarily in Southeast Asia and in focal areas of Latin America and Africa. It is the most common species affecting humans. *Paragonimus kellicoti* is occasionally found in North America.

Humans acquire the infection by ingesting metacercariae in raw, pickled, wine-soaked, or undercooked freshwater crabs or crayfish. The metacercaria excysts in the small intestine and burrows through the duodenal wall into the peritoneal cavity. It eventually penetrates the diaphragm and enters the lung. The host shows few symptoms during this migration but may exhibit intermittent coughing and chest pain. The parasite induces an inflammatory response in the lung characterized by the presence of neutrophils and eosinophils. Major symptoms associated with lung habitation are nonspecific and often include persistent cough, chest pain, and hemoptysis. Adults, which are reddish brown and approximately 1 cm long, live within capsules in the bronchioles.

Sputum is the primary diagnostic specimen. Eggs are expelled from the capsule into the bronchioles and carried upward in the sputum. Eggs may be found in feces if they have been coughed up and subsequently swallowed. *P. westermani* eggs are yellowish-brown, are broadly oval, are 80 to 115 $\mu\text{m} \times 48$ to 60 μm in size, and have a flattened operculum and slight shoulders. They are unembryonated when passed. The shell thickens at the end opposite the operculum (Fig. 28.52). These eggs can appear similar to those of *Diphylllobothrium latum* and must be carefully examined when seen in the feces. A wet mount of sputum demonstrates the egg in some patients.



Fig. 28.52 *Paragonimus westermani* egg (unstained, $\times 400$).

Blood flukes

Blood flukes, *Schistosoma* spp., differ from other flukes in the following manner:

- Both male and female forms exist; the female lives in an involution chamber, the gynecophoral canal, which extends the length of the male.
- The eggs are unoperculated.
- Humans are infected by direct cercarial penetration of skin.
- The flukes have a cylindric shape, rather than being dorsoventrally flattened.

The three primary species of schistosomes pathogenic to humans are *Schistosoma mansoni*, *Schistosoma haematobium*, and *Schistosoma japonicum*. Male adult schistosomes measure 7 to 20 mm, and adult females measure 7 to 26 mm. *S. mansoni*, which is commonly found in Africa, parts of South America, the West Indies, and Puerto Rico, lives in venules of the mesentery and large intestines. *S. japonicum*, which is commonly found in the Far East, including Japan, China, and the Philippines, lives in venules of the small intestine. This species, unlike the other two, has many mammalian reservoir hosts. *S. haematobium*, which is primarily found in the Nile Valley, the Middle East, and East Africa, lives in the veins surrounding the bladder. Two additional species pathogenic for humans are *S. intercalatum* and *S. mekongi*.

Clinical infections

Schistosomiasis (bilharziasis) affects approximately 200 million people worldwide. Symptoms are related to the phases of the fluke's life cycle and location of the adults. Cercarial penetration may cause a self-limiting local dermatitis, including irritation, redness, and rash, that persists for approximately 3 days. Larval migration through the body causes generalized symptoms, such as urticaria, fever, and malaise, which can last up to 4 weeks. The presence of the migrating larvae and adults in the veins causes little inflammatory damage seemingly because they acquire host HLAs and ABO blood group antigens on their surface that diminish the host's immune response.

Egg production and egg migration through the tissues are responsible for most of the immediate damage because the eggs are highly immunogenic. After release by the adult female, the eggs secrete enzymes and begin to penetrate

vessel walls and tissue. Eggs subsequently find their way into the lumen of the intestines or bladder. The egg spines cause trauma to the tissues and walls of the vessels during the early stage of acute infections and can result in gross or microscopic hematuria (*S. haematobium*) or diarrhea (*S. mansoni* and *S. japonicum*). In some individuals, especially those heavily infected with *S. japonicum*, an acute serum sickness-like illness (Katayama fever) occurs during the initial egg-laying period. This is induced by hypersensitivity response to egg antigens and is characterized by increased circulating levels of immune complexes and eosinophils.

In chronic infections, the eggs remaining in the tissue induce an immune response, resulting in granuloma formation, which leads to thickening and fibrotic changes. Scarring of the veins, development of ascites, pain, anemia, hypertension, hepatomegaly, and splenomegaly are also seen. In urinary schistosomiasis, microscopic bleeding into urine is present during the acute phase. In chronic stages, dysuria, urine retention, and urinary tract infections occur.

Penetration of humans by cercariae of the flukes of birds and other mammals causes schistosomal dermatitis, commonly referred to as *swimmer's itch*. Foreign proteins from these cercariae elicit a tissue reaction characterized by small papules 3 to 5 mm in diameter, edema, erythema, and intense itching. Symptoms last about 1 week and disappear as cercariae die and degenerate.

Life cycle

The life cycles of all three schistosomes are identical (Fig. 28.53). The eggs are embryonated when excreted, and the miracidium is released when the egg reaches water. After

the miracidium penetrates a snail (the first intermediate host), sporocysts and then cercariae are produced during a 6-week period. Cercariae migrate from the snail into water. Cercariae, with the help of enzymes, penetrate intact human skin. Once in the vasculature, they shed their forked tails and are referred to as *schistosomula*. They circulate until they reach the lungs or enter the liver, where maturation and pairing of the female and male are completed. The adults use oral and ventral suckers for attachment. The paired adult flukes use the portal system to reach veins of the intestine or bladder. Adults living in the veins can be killed with praziquantel, but the drug has no effect on the eggs in tissues.

Laboratory diagnosis

Diagnosis is made by finding embryonated eggs in feces (*S. mansoni* and *S. japonicum*) or in urine (*S. haematobium*). The egg of *S. mansoni* (Fig. 28.54A) is yellowish, elongated, 115 to 175 $\mu\text{m} \times 45$ to 75 μm in size, and has a prominent lateral spine. *S. haematobium* eggs (see Fig. 28.54B) are elongated, 110 to 170 $\mu\text{m} \times 40$ to 70 μm in size, and have a terminal spine. *S. japonicum* eggs (see Fig. 28.54C), which resemble *S. mekongi* eggs, are round, 60 to 95 $\mu\text{m} \times 40$ to 60 μm in size, and have a small, curved, rudimentary spine that might be obscured. The best time to collect eggs in urinary schistosomiasis is during peak excretion time in the early afternoon (noon to 2 PM). Biopsy may also be used in the diagnosis of schistosomiasis. Serodiagnosis can be useful to diagnose infection in patients from nonendemic countries who develop symptoms after visiting endemic areas. In areas of endemicity, serum antibody testing cannot differentiate between active and non-active infections. A urine-based, point-of-care, rapid test strip

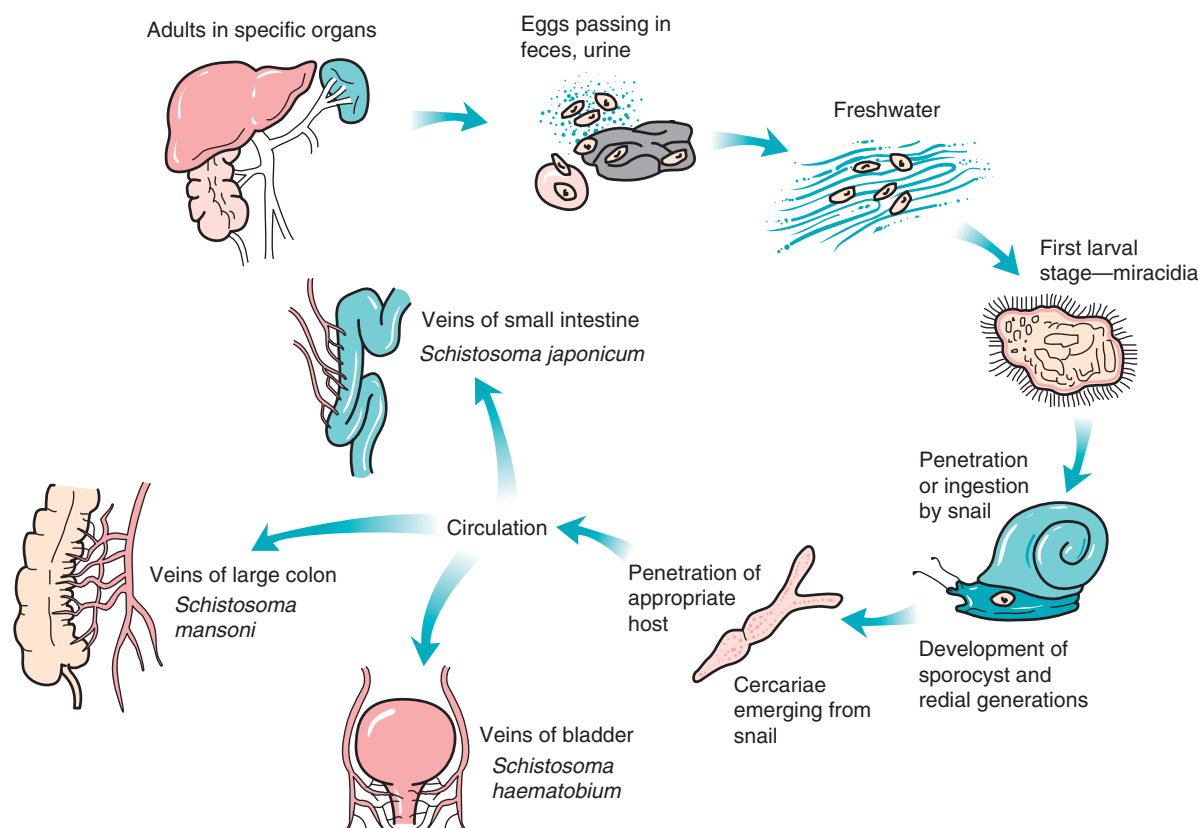


Fig. 28.53 Life cycle of blood flukes (*Schistosoma* spp.).

for the detection of parasite antigen (circulating cathodic antigen) is commercially available and useful for field screening in endemic areas.

Tapeworms

Tapeworms (cestodes) are another group of human parasites in the phylum Platyhelminthes. They show extensive size variation, ranging from 3 mm to 10 m, generally require intermediate hosts in their life cycle, and are hermaphroditic. They are ribbonlike organisms, whose method of growth involves the addition of segments, termed *proglottids*. Each proglottid, when mature, produces eggs infective for the intermediate host. Fig. 28.55 shows a general diagram of the tapeworm. The anterior headlike segment of a tapeworm, or **scolex**, has suckers and, in some species, hooklets as a means of attachment to the intestinal mucosa. The neck, located directly behind the scolex, is where proglottid production occurs. Treatment is targeted at detaching the scolex from the mucosa. Gravid proglottids at the distal end of the organism discharge eggs into feces.

Eggs of most of the tapeworms contain a **hexacanth embryo** or **oncosphere** (tapeworm embryo with three pairs of hooks that is infective for the intermediate host). Transmission to humans involves ingestion of a larval stage, called the **cysticercus** (larva consisting of a fluid-filled sac containing an invaginated scolex), cysticeroid, or plerocercoid larva (depending on the genus) in raw or undercooked meat or fish or of insects harboring the larval stage. The larval stage contains an invaginated scolex inside a protective membrane.

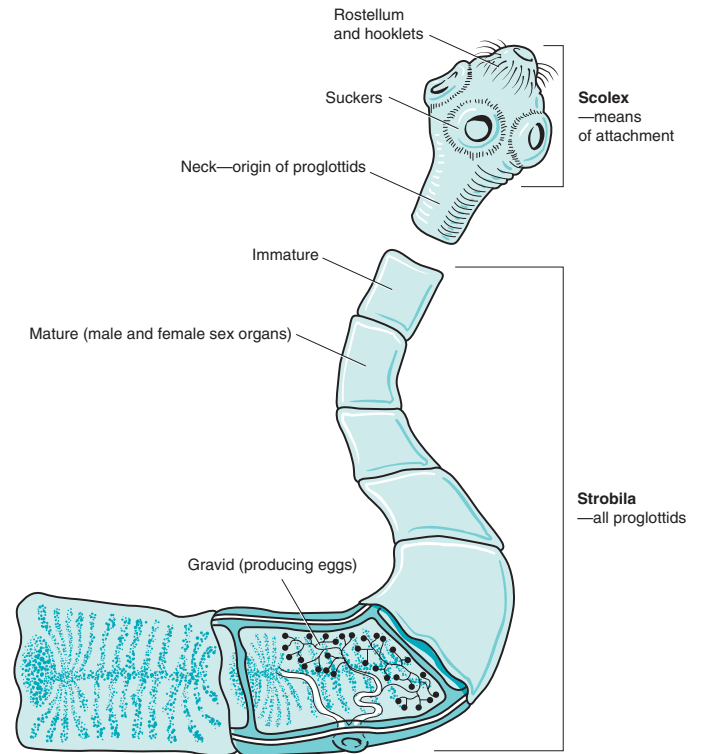


Fig. 28.55 A tapeworm.

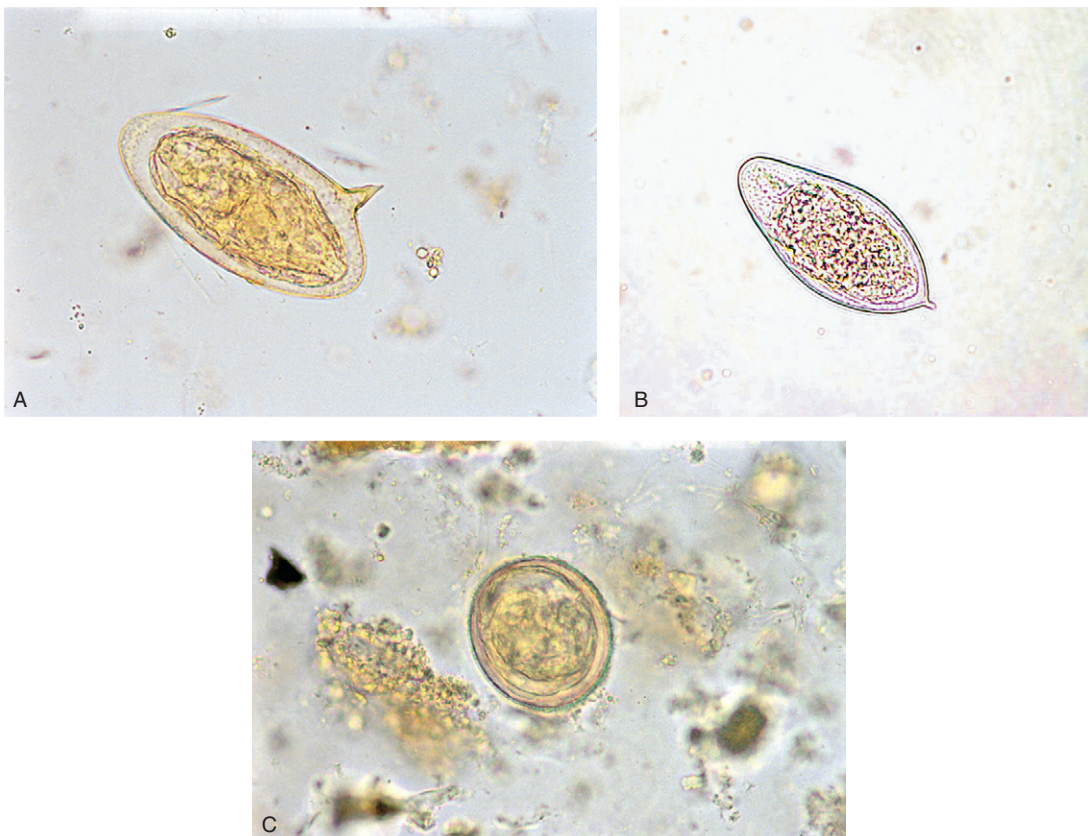


Fig. 28.54 A, *Schistosoma mansoni* egg. B, *Schistosoma haematobium* egg. C, *Schistosoma japonicum* egg. (Unstained wet mounts, $\times 400$.)

Table 28.8 Comparisons of tapeworm eggs

Organism	Average size (μm) and shape	Other identifying features
<i>Diphyllobothrium latum</i> 	58–76 $\times$ 40–50, oval	Inconspicuous operculum Small knob at end opposite operculum Unembryonated when passed
<i>Taenia</i> spp. 	30–35, round	Thick, brown, radially striated shell Embryonated, with six-hooked oncosphere when passed
<i>Hymenolepis nana</i> 	30–47, oval	Two membranes—inner has two polar knobs, from which four polar filaments extend into space between inner and outer membranes Embryonated, with six-hooked oncosphere when passed
<i>Hymenolepis diminuta</i> 	50–75, round to slightly oval	Two membranes—inner has very slight polar knobs No polar filaments Embryonated, with six-hooked oncosphere when passed
<i>Dipylidium caninum</i> 	20–40 (each egg), round; resembles <i>Taenia</i> spp.	Eggs passed in packet of 15–25 Eggs embryonated, with six-hooked oncosphere when passed

The diagnosis of tapeworm infection is usually made by finding eggs in feces, although proglottids can be used if they are passed intact. Intestinal tapeworm infections are usually treated with praziquantel; alternatively, they can be treated with niclosamide, albendazole, or nitazoxanide. Table 28.8 compares the characteristics of tapeworm eggs.

Diphyllobothrium latum

D. latum, the fish tapeworm, is found worldwide in areas in which the population eats pickled or raw freshwater fish. In the United States, it is primarily seen in the areas around the Great Lakes. Fish-eating mammals in endemic areas may also be infected. Humans usually harbor only a single worm, which attaches in the jejunum and can reach a length of up to 10 m. Most infected individuals demonstrate no clinical symptoms; others have vague GI symptoms, including nausea and vomiting and intestinal irritation. The organism may cause a vitamin B₁₂ deficiency, especially in persons of northern European descent, and long-term infection may lead to megaloblastic anemia.

The life cycle of *D. latum* is somewhat of a hybrid between that of the flukes and that of the tapeworms (Fig. 28.56). The operculated, unembryonated egg, passed in human feces, must reach water to mature. The first larval stage (coracidium) is ingested by a copepod and develops into a procercoid larva within the copepod. When the infected copepod is ingested by a fish, the larva leaves the fish's intestine and invades the flesh, where it develops into a plerocercoid larva, which consists of a scolex with a thin, ribbonlike portion of tissue. Humans ingest the plerocercoid larvae by eating raw or undercooked fish. The scolex is released in the intestine, where it develops into an adult worm.

The scolex, proglottid, and egg are diagnostic structures that can be found in fecal specimens. The egg, however, is usually detected. The egg is unembryonated when passed, operculated, and yellow to brown (Fig. 28.57). It is about 58 to 76 $\mu\text{m} \times$ 40 to 50 μm in size and has a small, knoblike protuberance at the end opposite the operculum. The knob may not be seen on all eggs, so size and lack of shoulders must be used to distinguish the egg from that of *Paragonimus westermani*. The proglottid is wider than it is long, with a characteristic rosette-shaped or coiled uterus. The scolex, which is 2 to 3 mm long, is elongated and has two sucking grooves, one located on the dorsal surface and the other on the ventral surface, and no hooks.

Taenia

Two *Taenia* spp. infect humans: (1) *Taenia saginata*, the beef tapeworm, which is found primarily in beef-eating countries of the world, and (2) *Taenia solium*, the pork tapeworm, which is found in areas of the world with a high consumption of pork, such as Latin America. Both organisms attach to the intestinal mucosa of the small intestine. The adult *T. saginata* can reach a length of 10 m, whereas the adult *T. solium* may reach only 7 m. Infection with the adult tapeworm of either species usually causes few clinical symptoms, although vague abdominal pain, indigestion, and loss of appetite may be present. The major complication of infection with *T. solium* is cysticercosis, in which the infected individual becomes the intermediate host and harbors the larvae in tissues. This is discussed in the section on tissue infections with cestodes (see the "Cysticercosis" section later in this chapter).

As noted, the life cycles of the two *Taenia* spp. (Fig. 28.58) are identical except that humans may also serve as intermediate

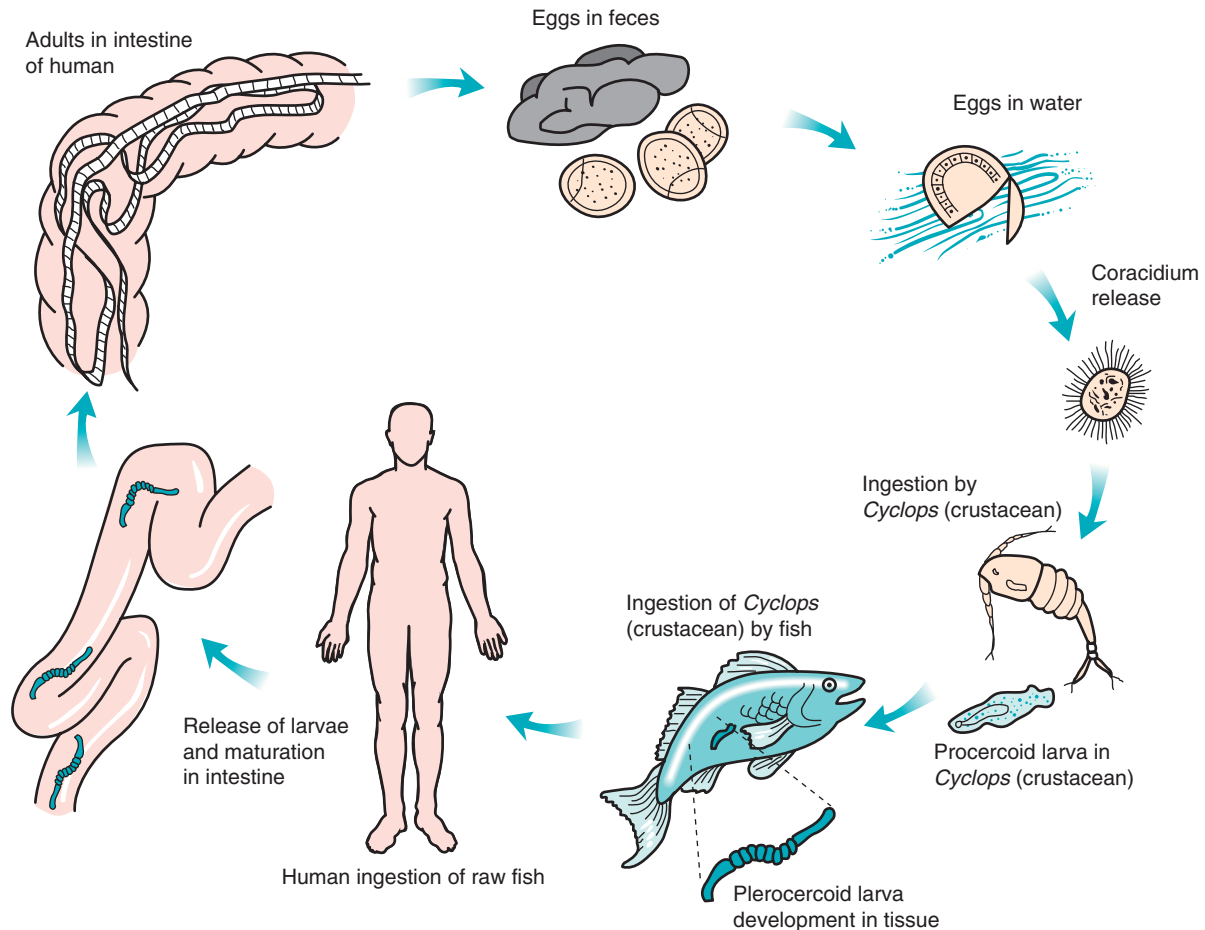


Fig. 28.56 Life cycle of *Diphyllbothrium latum*.

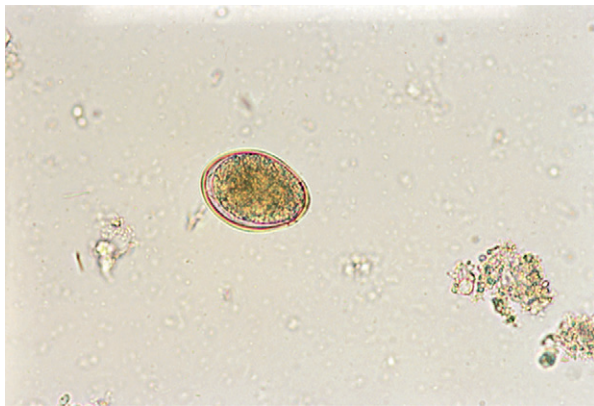


Fig. 28.57 *Diphyllbothrium latum* egg (unstained, $\times 400$).

hosts for *T. solium*. Embryonated eggs are passed in human feces and ingested by the intermediate host. The oncosphere is freed in the intestinal tract, migrates through the intestinal wall, and gains access via the circulatory system to the muscles of the host, where it transforms into a cysticercus. When humans ingest raw or undercooked meat, the scolex in the cysticercus is freed, attaches in the human small intestine, and matures into the adult tapeworm within 10 weeks. The proglottids are motile and, if broken off in the intestinal tract, may actively migrate out via the anus.

Laboratory diagnosis of *Taenia* infection can be made by finding the egg, scolex, or proglottid in the feces. The egg, which is the most common stage found, is yellow to brown, round, and surrounded by a thick wall with radial striations; it measures 30 to 35 μm in diameter. The egg is embryonated, with a six-hooked oncosphere when passed in feces (Fig. 28.59). Eggs of these species are indistinguishable and must be reported as *Taenia* sp. eggs. Gravid proglottids may be seen in the stool specimen and can be used to differentiate the two species. Proglottids of *T. solium* have 7 to 13 primary uterine branches on each side of the main uterine trunk, whereas proglottids of *T. saginata* show 15 to 20 per side. The scolex, if found, can also be used to distinguish the organisms. The scolex of *T. saginata* is less than 5 mm long and has four suckers, whereas that of *T. solium* has a rostellum, with a double row of 25 to 30 hooklets in addition to the four suckers.

Hymenolepis

The dwarf tapeworm, *Hymenolepis nana*, is found worldwide and is a common tapeworm in children, whereas *Hymenolepis diminuta*, the rat tapeworm, is seen less frequently. Light infections are usually asymptomatic; large numbers of worms may cause abdominal pain, diarrhea, irritability, and headache. Infections with *H. nana* are easily transmitted among children because an intermediate host is not required. Direct fecal-oral transmission of the egg, development of the cysticercoid in

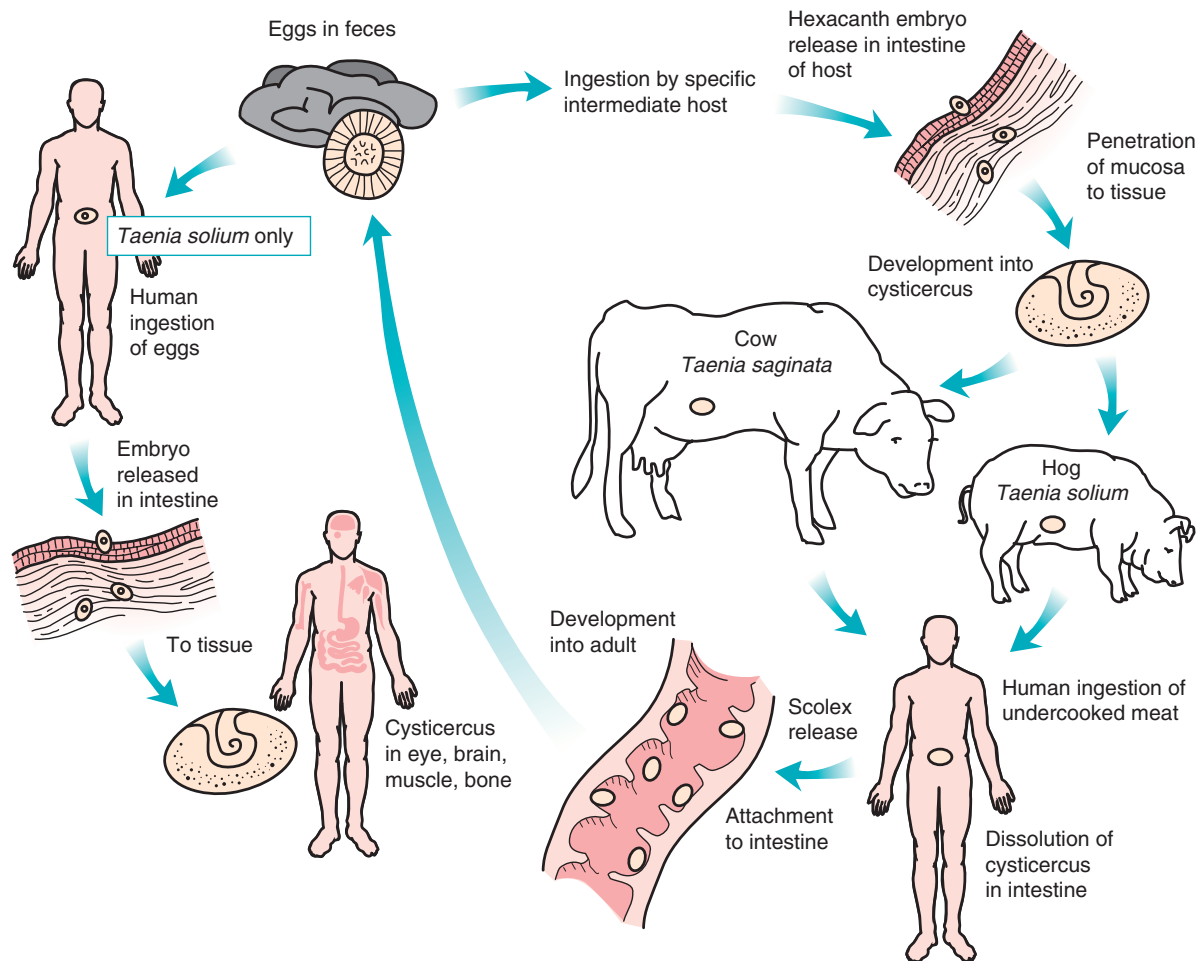


Fig. 28.58 Life cycle of *Taenia* spp.

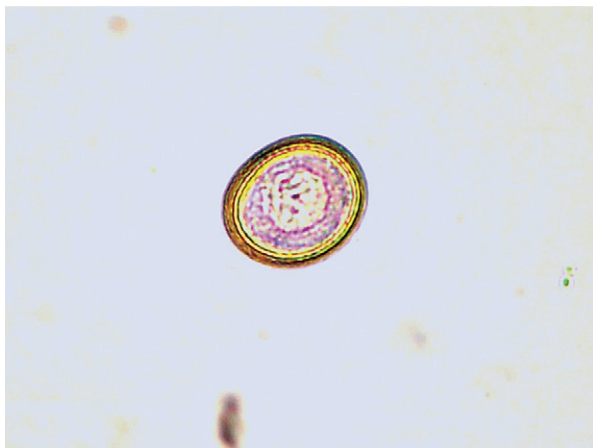


Fig. 28.59 *Taenia* sp. embryonated egg (unstained wet mount, $\times 400$).

the intestinal tissue of the host, and reentry into the lumen for development into an adult characterize the life cycle (Fig. 28.60). This autoinfective life cycle is most common, although an insect vector may serve as an intermediate host in an alternative form of the life cycle.

The adult *H. nana* is approximately 40 mm long and has a small scolex, with four suckers and a rostellum with spines.

The primary method of diagnosis is finding the egg in a stool specimen. The egg is spherical to oval, measures 30 to 47 μm , and has a grayish color. The hexacanth embryo is contained within an inner membrane, and the area between the inner membrane and egg wall contains two polar thickenings from which four to eight polar filaments extend (Fig. 28.61). Infection with *H. diminuta* is acquired by ingesting fleas that contain the infective cysticercoid. The adult tapeworm is 20 to 60 cm long. The egg, which must be distinguished from that of *H. nana*, is 50 to 75 μm , gray or straw colored, and oval. An inner membrane with inconspicuous polar thickenings but no polar filaments surround the oncosphere (Fig. 28.62).

Dipylidium caninum

Humans serve as accidental hosts for *Dipylidium caninum*, the dog tapeworm. Children are usually infected by ingesting fleas containing the larval stage. The resulting infections generally do not cause any symptoms. The proglottid may be seen in human feces and is characterized by its pumpkin-seed shape, twin genitalia, and the presence of two genital pores, one on each side of the proglottid. The eggs are characteristically seen in packets of 15 to 25 eggs. Individual eggs are 20 to 40 μm in size and resemble those of *Taenia* spp. (Fig. 28.63).

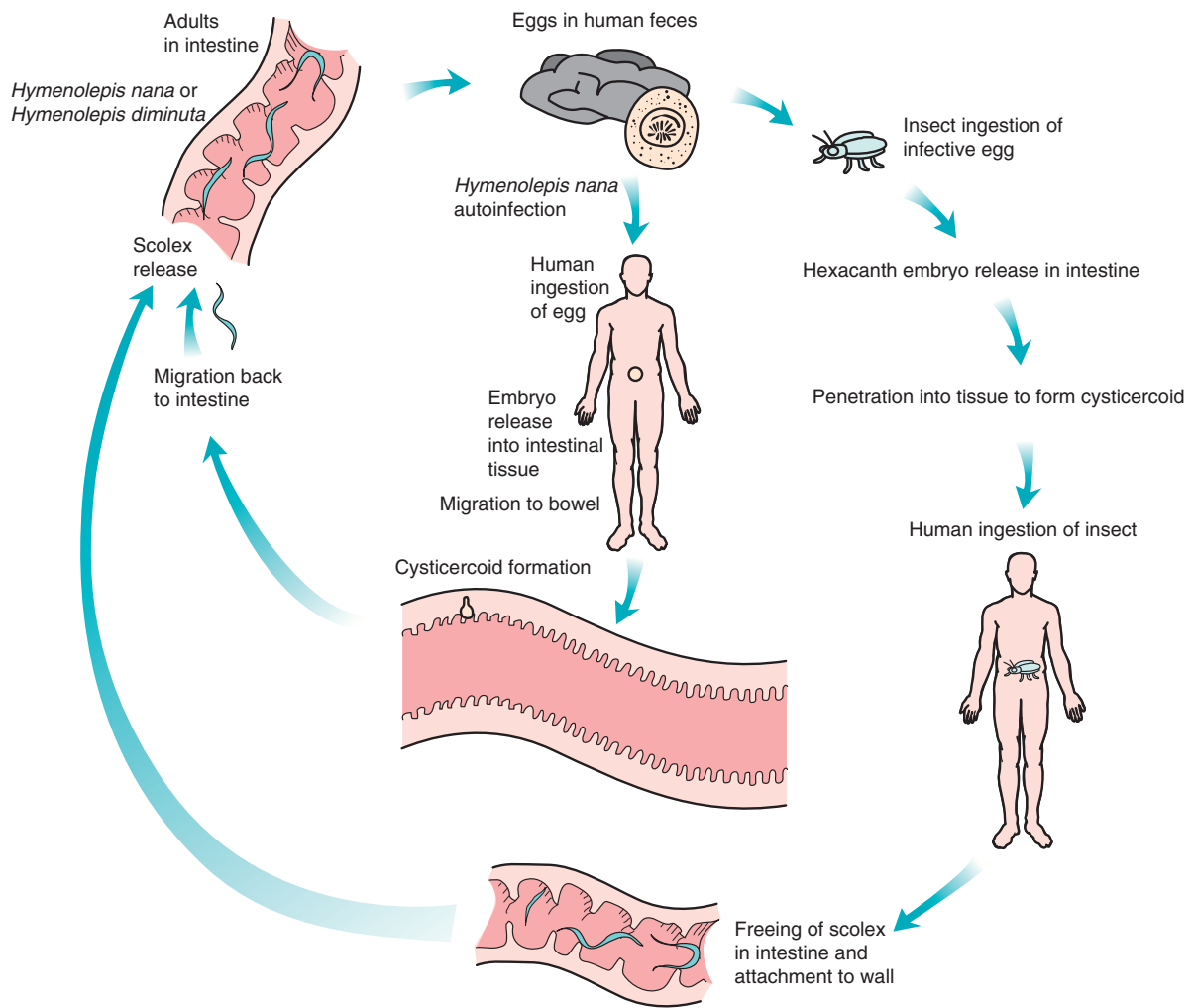


Fig. 28.60 Life cycle of *Hymenolepis* spp.

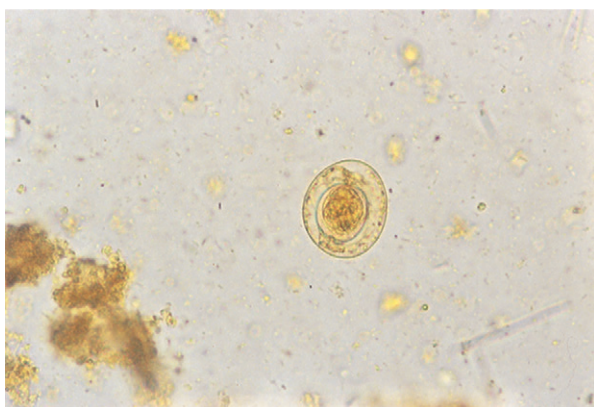


Fig. 28.61 *Hymenolepis nana* egg (iodine wet mount, ×400).

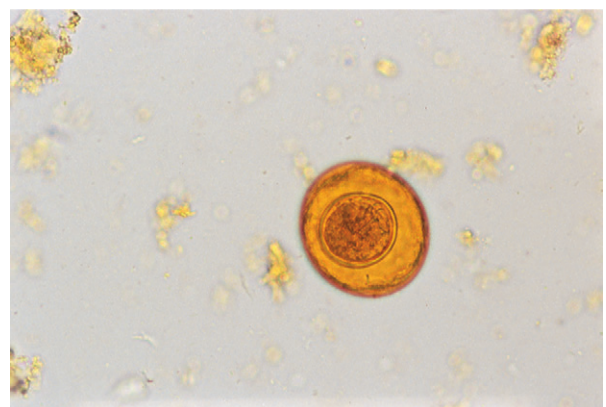


Fig. 28.62 *Hymenolepis diminuta* egg (iodine wet mount, ×400).

Tissue infections with cestodes

Cysticercosis, sparganosis, and hydatid cyst disease are the major diseases caused by the tissue stage of a tapeworm. They originate when a human accidentally becomes the intermediate host for the parasite.

Cysticercosis

Cysticercosis results when a human ingests the infective eggs of *T. solium*, the pork tapeworm, thus becoming an intermediate host. The disease is endemic in areas of rural Latin America, Asia, and Africa and is reemerging as a zoonosis in the United States as a result of immigration of persons from

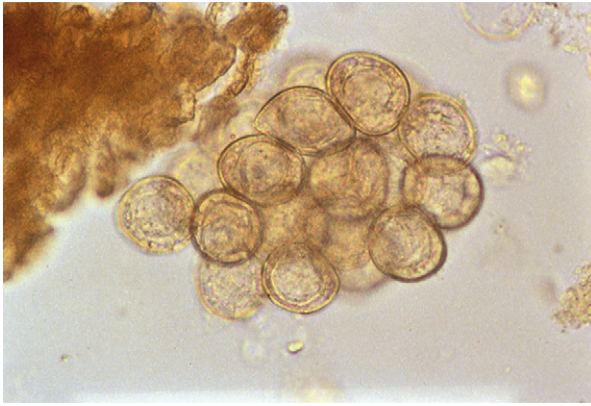


Fig. 28.63 *Dipylidium caninum* egg packet (unstained, $\times 400$).

endemic areas. Contributing factors for infection include poor hygiene and sanitary habits that result in ingestion of food containing an infective egg as well as residence in rural areas and hog farming. Once the egg is ingested, the hexacanth embryo is released into the intestines, penetrates the intestinal wall, and enters the circulation to develop as a cysticercus in any tissue or organ. The larva can live up to 7 years and elicits a host tissue reaction, resulting in production of a fibrous capsule. Once the organism dies and releases larval antigens, there is an intense host inflammatory reaction that leads to tissue damage in the area. Eventual calcification of the cysticercus will occur. The most commonly infected sites are the striated muscle, eye, and brain.

Light infections usually cause no clinical symptoms. When present, symptoms depend on the organ affected and the size and number of cysticerci present. Muscular pain, weakness, and cramps characterize infections of the striated muscle. A cysticercus can form in the vitreous or subretinal space of the eye. Retinal detachment, intraorbital pain, flashes of light, and blurred vision may occur. Neurocysticercosis, which is the most serious manifestation, is the causative agent for up to 30% of epilepsy cases seen in patients in countries where the disease is endemic. In the United States, the condition is often seen in Hispanic immigrants from endemic areas. Infection may be manifested by headaches, symptoms resembling those seen in meningitis or a brain tumor, convulsions, or a variety of motor and sensory problems.

The cysticercus is oval, translucent, and about 5 to 18 mm in size. It contains an invaginated scolex containing four suckers and a circle of hooklets on the rostellum. The infection may be diagnosed by various methods, including radiography to detect calcified cysts, ophthalmoscopic examination of the eye to detect cysticerci, imaging techniques (CT and MRI) to locate larvae in the brain, and biopsy and histologic staining of tissue. Serologic tests, including immunoblot techniques, have been developed to detect antibodies against specific cysticercal antigens. These are most useful in epidemiologic studies.

Sparganosis

Human infection with the plerocercoid larva (sparganum) of a dog or cat tapeworm can result in sparganosis. Humans acquire the infection by ingesting a copepod containing the proceroid larva; by ingesting reptiles, amphibians, or other

animals containing the plerocercoid larva; or through invasion by the plerocercoid larva when the raw tissue from the second intermediate host is used as a poultice. The disease is most common in Southeast Asia. Infection is often seen in the eye after a poultice has been applied to relieve an infection. The organism may also cause migratory subcutaneous nodules, itching, and pain. The diagnosis of sparganosis is made by finding a small, white, ribbonlike organism with a rudimentary scolex. Size ranges from a few millimeters to 40 cm. The organism may be removed surgically.

Echinococcosis

Echinococcosis (hydatid cyst disease) is an infection by *Echinococcus granulosus* that normally involves the dog or another member of the family Canidae as the definitive host. Sheep and other herbivores are the usual host of the larval stage (hydatid cyst). The disease is primarily seen in sheep-raising areas of the world, including Australia, southern South America, and parts of the southwestern United States.

The adult worm is approximately 5 mm long and contains only three proglottids. The eggs are found in the feces of dogs or other definitive hosts and resemble those of *Taenia* spp. A human becomes an intermediate host by accidentally ingesting the eggs of *E. granulosus* containing the hexacanth embryo. The oncosphere is liberated in the intestine, penetrates the mucosa, enters the circulation, and usually lodges in the liver. The embryo develops a central cavity-like structure lined with a germinal membrane, from which brood capsules and protoscolices (hydatid sand) develop. The hydatid cyst's size is limited by the organ in which it develops. In bone, a limiting membrane never develops, so the cyst fills the marrow and eventually erodes the bone.

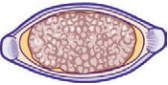
Symptoms differ, depending on the organ infected. Pressure from the increasing size of a cyst may cause necrosis of surrounding tissue. Rupture of the cyst liberates large amounts of foreign protein (allergen) that may elicit an anaphylactic response. In addition, freed germinal epithelium may serve as a source of new infection. The diagnosis can be made by radiologic examination, ultrasonography, or other imaging techniques. Aspiration of the cyst contents usually reveals the presence of protoscolices.

Roundworms

Human roundworms include those that infect the intestinal tract and blood and tissue. These organisms, found worldwide, are transmitted by the ingestion of the embryonated egg or by direct penetration of skin by larvae in the soil, or they may require an insect vector. Intestinal roundworms are the most common of all the helminths that cause human infections in the United States. Infected individuals are found in highest numbers in warm, moist areas of the Southeast and in areas with poor sanitation.

Roundworms are characterized by the presence of two sexes and a life cycle that may involve larval migration throughout the body. The adults obtain nourishment by absorbing nutrients from partly digested intestinal contents or by sucking blood. Patients may be asymptomatic or symptomatic; the severity of the symptoms is related to the worm burden, host's nutritional status and age, and duration of

Table 28.9 Comparisons of intestinal roundworm eggs and larvae

Organism	Average size (μm) and shape	Other identifying features
<i>Ascaris lumbricoides</i> Fertile 	45–75 $\times$ 35–50, oval	Bile-stained shell Bumpy, mammillated In one-cell stage when passed Some eggs may be decorticated (lack mammillated coat)
Infertile 	85–95 $\times$ 43–47, oval (some bizarrely shaped)	Mammillated Thin shell Undifferentiated internal granules
<i>Enterobius vermicularis</i> 	50–60 $\times$ 20–30, oval, flattened on one side	Colorless shell Usually embryonated with C-shaped larva
<i>Trichuris trichiura</i> 	50–55 $\times$ 22–23, barrel shaped	Bile-stained, thick shell Hyaline polar plugs Unembryonated when passed
Hookworm egg 	50–60 $\times$ 35–40, broadly oval	Thin shell, colorless In four- to eight-cell stage when passed
Rhabditiform larva 	250–300	Long buccal capsule Inconspicuous genital primordium
Filariform larva 	500	Pointed tail Esophageal-to-intestinal ratio 1:4
<i>Strongyloides stercoralis</i> Rhabditiform larva 	Egg rarely seen; resembles that of hookworm 200–250	Short buccal capsule Prominent genital primordium
Filariform larva 	500	Notched tail Esophageal-to-intestinal ratio 1:1

infection. Most roundworm infections can be treated with oral administration of albendazole or mebendazole. Table 28.9 compares the diagnostic characteristics of the eggs and larvae of intestinal roundworms.

Enterobius vermicularis

E. vermicularis, often called the *pinworm*, is a worldwide parasite commonly detected in children, especially those 5 to 10 years of age. It is estimated that 20 million to 40 million individuals are infected in the United States alone. Key risk factors for this infection are inadequate personal and community hygiene. Enterobiasis is frequently found in families, kindergartens, daycare centers, or crowded conditions in which the eggs can be easily transmitted. The eggs are resistant to drying and are easily spread in the environment. Adult worms live in the large intestine (cecum), although they have occasionally

been found in the appendix or vagina. Ectopic infections have also caused endometritis, urethritis, and salpingitis. There is evidence that the organism may also be associated with urinary tract infections in young girls.

Although patients with *E. vermicularis* infection are often asymptomatic, they may experience loss of appetite, abdominal pain, loss of sleep, and nausea and vomiting. Anal pruritus is caused by migration of the female worm to the perianal area. The life cycle of this organism (Fig. 28.64) is characterized by migration of the female out through the anus at night to lay eggs in the perianal area. The eggs are infective with a third-stage larva within several hours of being laid. Typically, transmission involves inhalation or ingestion of the infective eggs. Direct anal-oral transmission occurs in children as a result of poor hand washing or fingernail biting. Autoinfection, in which the hatched larvae reenter the intestine to mature into an adult, may also occur.

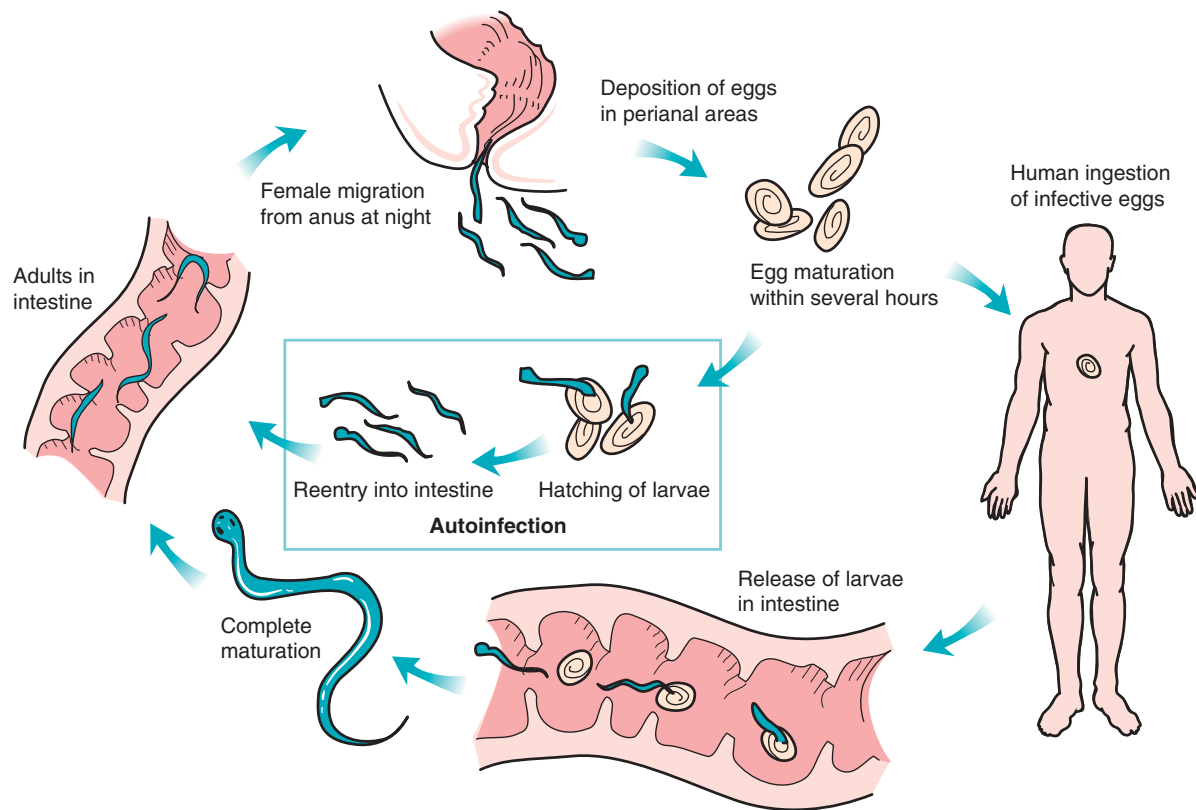


Fig. 28.64 Life cycle of *Enterobius vermicularis*.

Treatment for pinworm includes mebendazole, pyrantel pamoate, or albendazole. Treatment requires an initial dose followed by an additional treatment 2 weeks later to prevent reinfection caused by ingestion of eggs remaining in the environment.

Because eggs are laid outside the body in the perianal area and are rarely present in the stool, a fecal specimen is unsatisfactory for diagnosis. The cellophane tape preparation or commercially available sticky paddle is considered the diagnostic method of choice. The procedure must be done as soon as the child arises in the morning. The perianal area is touched with the sticky side of the tape or paddle. The adult female can occasionally be seen in this preparation. Because the gravid female can migrate into the vagina, eggs can also be seen in vaginal specimens.

The adult female measures 8 to 13 mm long and has a long, pointed tail and three cuticle lips, with alae at the anterior end. The less commonly seen male is 2 to 5 mm long, with a curved posterior. The egg is oval, colorless, and slightly flattened on one side. It measures approximately 50 to 60 μm $\times$ 20 to 30 μm . The egg is usually seen embryonated, with a C-shaped larva (Fig. 28.65).

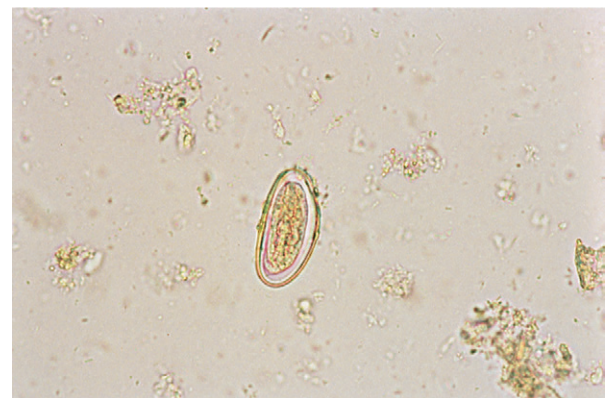


Fig. 28.65 *Enterobius vermicularis* egg (unstained, $\times 400$).

Trichuris trichiura

Ascaris lumbricoides, *T. trichiura*, and two genera of hookworms, *Ancylostoma* and *Necator*, are the most common soil-transmitted helminths. These organisms have a worldwide distribution and are major causes of morbidity, rather than death, in developing areas of the world. Estimates indicate that 25% of the world's population is infected with

one or more of these organisms. Chronic infection caused by these helminths, especially hookworm, can adversely affect physical and mental development in children. A heavy worm burden is more likely to result in complications. Some of the risk factors for infection include poor sanitation (personal and community), poverty, occupation, and climate (necessary for maturation and survival of the eggs in the soil). *Strongyloides stercoralis* is also a soil-transmitted helminth but does not have the broad geographic distribution of the other organisms.

T. trichiura, referred to as the *whipworm*, is found worldwide, especially in areas with a moist, warm climate. It is found in the southeastern United States, often as a co-infection



Fig. 28.66 *Trichuris trichiura* egg (unstained, $\times 400$).

with *A. lumbricoides*. Light infections with *T. trichiura* rarely cause symptoms; heavy infections result in intestinal bleeding, weight loss, abdominal pain, nausea and vomiting, and chronic diarrhea. As the adults thread themselves through the intestinal mucosa, inflammation develops. Prolonged heavy infection can result in colitis or diarrhea with blood-tinged stools. Rectal prolapse can be the result of repeated heavy infections in undernourished children. Hypochromic anemia may occur in children with inadequate iron and protein intake in the presence of constant, low-level bleeding with chronic infection. Treatment includes mebendazole or albendazole.

Eggs are passed in feces and require at least 14 days in warm, moist soil for embryonation to occur. Humans acquire infection by ingesting the infective egg. The larva is released in the small intestine and undergoes several molts before maturing into an adult worm in the cecum. The egg and occasionally the adult of *T. trichiura* may be seen in fecal specimens. The adult male measures 30 to 45 mm and has a thin anterior and a thick, coiled posterior. The female is 30 to 50 mm long, with a thin anterior and thick, straight posterior. Eggs are the typical diagnostic form. They are brown, barrel-shaped, unembryonated when passed, and 50 to 55 $\mu\text{m} \times 22$ to 23 μm in size, with a thick wall and hyaline polar plugs at each end (Fig. 28.66).

Ascaris lumbricoides

An estimated 1 billion people worldwide are infected with *A. lumbricoides*. The organism is most common in tropical and subtropical areas, and in areas of poor sanitation. Children are most commonly infected. Infections are uncommon in the United States, but the organism is most frequently seen in rural parts of the Southeast. Transmission is primarily by the fecal-oral route, and clinical symptoms may be related to the different phases of the life cycle. The organism is often found concurrently with whipworm.

Abdominal discomfort, loss of appetite, and colicky pains are caused by the presence of adult worms in the intestine. There is evidence that heavy infections may contribute to lactose intolerance and malabsorption of some vitamins, including vitamin A. In children, large numbers of adult worms can cause intestinal obstruction. Because the worms feed on liquid intestinal contents, chronic infection with *A. lumbricoides*

in children may hamper growth and development. Larvae migrating through the lungs can cause an immune response in the host characterized by asthma, edema, pneumonitis, and eosinophilic infiltration. Rarely, larvae are seen in the sputum in heavy infections. Occasionally, fever or other disease conditions cause the adults to migrate from the intestine and invade other organs, resulting in peritonitis, liver abscess, or secondary infection in the lungs. Adults may also exit through the mouth, tear duct, or nose and have been reported to enter and block catheters. Eosinophilia may be present. Albendazole or mebendazole are the drugs of choice for treating infection with *Ascaris*.

Eggs that are deposited in warm, moist soil become infective within about 2 weeks. After the egg is ingested, larvae hatch in the duodenum, penetrate the intestinal wall, and gain access to the hepatic portal circulation. They break out from the capillaries into the lungs, travel up the bronchial tree and trachea and over the epiglottis, and are swallowed. Maturation is completed in the intestine. The life cycle (Fig. 28.67) takes about 50 days after infection until adults are mature. The usual diagnostic stage is the egg. Fertile *Ascaris* eggs are oval, measure 45 to 75 $\mu\text{m} \times 35$ to 50 μm , and have a thick hyaline wall surrounding a one-cell-stage embryo. Most eggs have a brown, bile-stained, mammillated outer layer (Fig. 28.68). Some eggs, described as *decorticated*, lack the mammillated outer coat. Infertile eggs, up to 90 μm in length, are often elongated and contain a mass of highly refractile granules. Adults, measuring 15 to 35 cm long and about the diameter of a lead pencil, may be seen in stool samples. The female has a straight posterior, and the male has a curved posterior. Both have three anterior lips with small, toothlike projections.

Hookworms

Worldwide, about 740 million people are estimated to be infected with hookworms. The greatest incidence is in the tropics and subtropics, particularly Asia, Latin America, the Caribbean, and sub-Saharan Africa. In the United States in the 1950s, hookworm infection was very common, particularly in the South. Improved sanitation has greatly reduced the incidence. Unlike other helminths, in which infection peaks in childhood and adolescence, the hookworm burden often increases with age. Classically, two species of hookworm, *Necator americanus* (New World) and *Ancylostoma duodenale* (Old World), were known to infect humans. Recently an animal parasite, *Ancylostoma ceyanicum* (prevalent in Southeast Asia, Australia, and the Pacific Islands) was recognized as a human pathogen. *A. duodenale* is seen in southern Europe and northern Africa along the Mediterranean as well as in parts of Southeast Asia and South America. *N. americanus* has a geographic distribution in Africa, Southeast Asia, and South and Central America and is endemic in rural areas of the southeastern United States.

Adults of the three species can be differentiated by the morphology of the buccal capsule or, in the male, the copulatory bursa. The eggs, however, are identical. These worms live in the small intestine and attach to the mucosa by means of teeth (*A. duodenale*) or cutting plates (*N. americanus*). They digest the tissue plug and pierce capillaries. Once attached, they continue to ingest blood as a source of nourishment by secreting anticoagulants, platelet inhibitors, and substances

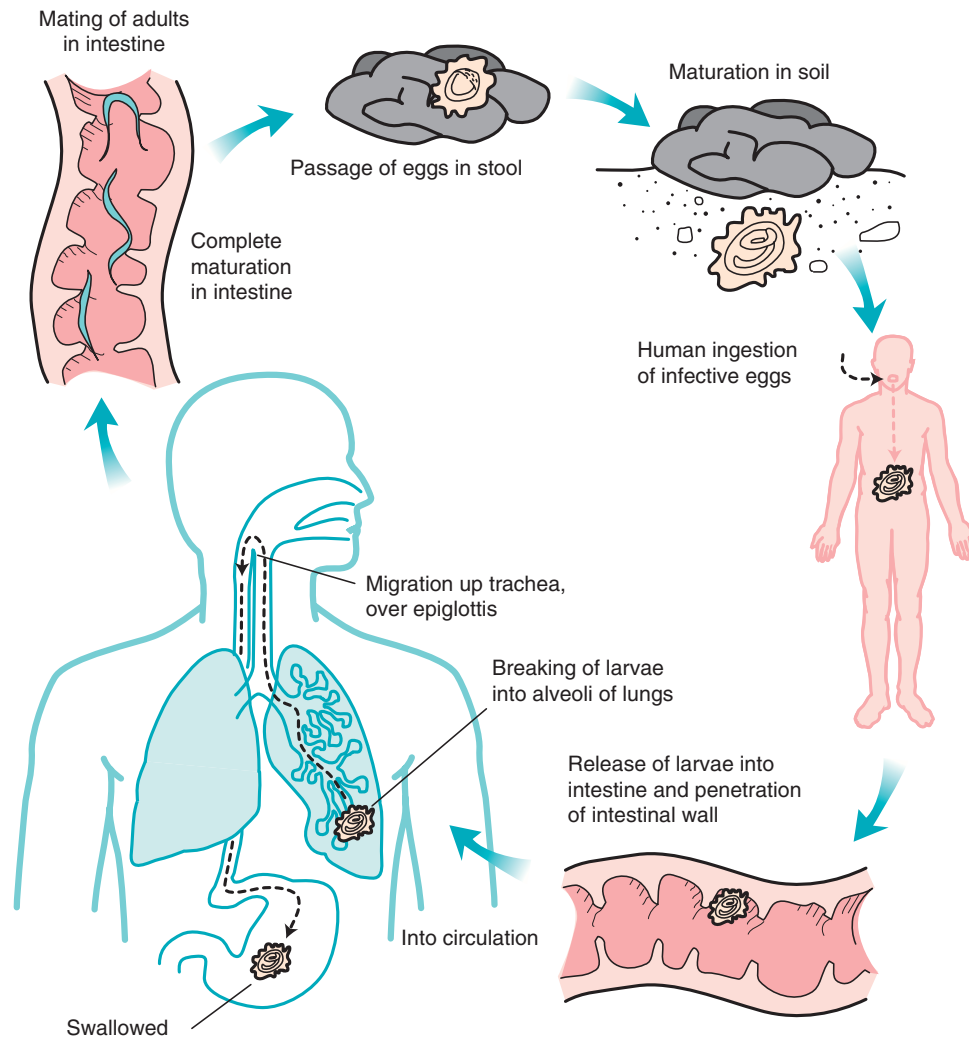


Fig. 28.67 Life cycle of *Ascaris lumbricoides*.

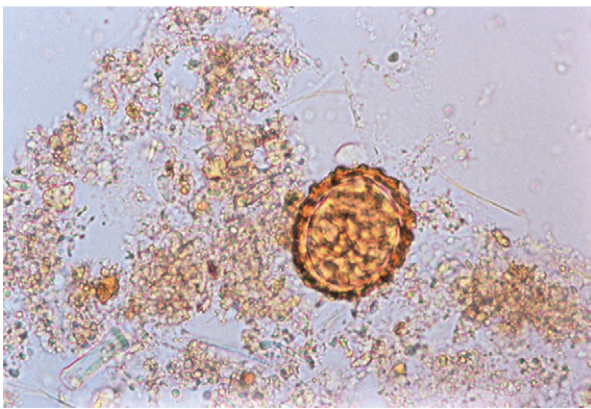


Fig. 28.68 *Ascaris lumbricoides* egg, fertile (unstained, $\times 400$).

that interfere with the tissue factor–factor VIIa complex. The organisms also secrete substances that interfere with the action of digestive enzymes and inhibit host absorption of nutrients.

Clinical symptoms differ according to the phase of the life cycle and the worm burden. A small, red, itchy papule,

referred to as *ground itch*, develops at the site of larval penetration. If large numbers of larvae are present during the lung phase of migration, the patient may have bronchitis, but unlike with *Ascaris* larvae, no host sensitization occurs. The most severe symptoms are associated with the adult, including nonspecific symptoms such as diarrhea, fever, and nausea and vomiting. Eosinophilia is often present. A few patients may experience pica and then ingest dirt (geophagia).

Blood loss, ranging from 0.03 to 0.2 mL per worm per day, is primarily the result of the ingestion of blood by the adult worm. Hemorrhages at the site of attachment, however, also contribute to total blood loss. Chronic heavy hookworm infection can lead to microcytic hypochromic anemia, especially in children whose diet is inadequate in iron and protein. A chronic heavy infection may affect the mental and physical development of a child because of the complications of anemia and malnutrition. Infection is usually treated with albendazole or mebendazole. Supportive therapy, including iron and protein supplements, may be needed in severe cases, especially if the child shows evidence of anemia or if the infection occurs in a pregnant woman. Vaccine development using hookworm antigens is being targeted as a way to help control infections.

Case check 28.2

The patient in the Case in Point shows several characteristics associated with hookworm infection. The vesicular lesions on the foot (ground itch) represent sites at which filariform larvae have penetrated skin and entered the blood circulation. In the case of other roundworms, the egg is usually ingested; in the case of tapeworms, there could possibly be a history of eating raw or undercooked meat.

The presence of a low hemoglobin level and microcytic hypochromic anemia is common in severe or long-term hookworm infection because the organism attaches to the intestinal mucosa and uses blood as a source of nourishment. The long-term, low-level blood loss will cause this type of anemia, characteristic of iron-deficiency anemia, in individuals who are malnourished or lack adequate dietary iron.

When the eggs are deposited in warm, moist soil, the non-infective, feeding, first-stage **rhabditiform larva** develops within 1 to 2 days and feeds on bacteria in the soil. A non-feeding, infective, filariform larva develops within 1 week. Humans are infected when the filariform larvae penetrate skin. The organisms enter the circulation and break out of the capillaries into the lung and then migrate up the bronchial tree, over the epiglottis, and into the digestive tract. After additional larval molts, the worms attach to the mucosa in the small intestine. Eggs are produced within 6 to 8 weeks

after skin penetration by the filariform larva. Fig. 28.69 shows the life cycle of the hookworm.

Adult hookworms are rarely seen in stool specimens; the egg and the rhabditiform larva are the usual diagnostic stages. The eggs and rhabditiform larvae of the three species are indistinguishable; therefore the laboratory report can only state "hookworm" when a characteristic egg or larva is found in a stool specimen. The egg is oval, colorless, thin shelled, and 50 to 60 μm long and usually contains an embryo in the four- to eight-cell stage of cleavage (Fig. 28.70). The rhabditiform larva must be differentiated from that of *S. stercoralis* because treatment is different. The hookworm rhabditiform larva is 250 to 300 μm long and has a small, inconspicuous, genital primordium (Fig. 28.71A) and a long buccal capsule (see Fig. 28.71B). The filariform larva also must be distinguished from that of *S. stercoralis*. Hookworm filariform larvae are about 500 μm long, with a pointed tail and an esophageal-intestinal ratio of 1:4.

Strongyloides stercoralis

S. stercoralis, known as the *threadworm*, inhabits the small intestine but is also capable of existing as a free-living worm. It is endemic in the tropics and subtropics, including Southeast Asia, Latin America, and sub-Saharan Africa but has been found on all continents except Antarctica. It is estimated that 30 million to 100 million people are infected worldwide. *S. stercoralis* can persist in the host for decades after initial infection, and infection may progress to hyperinfection if the

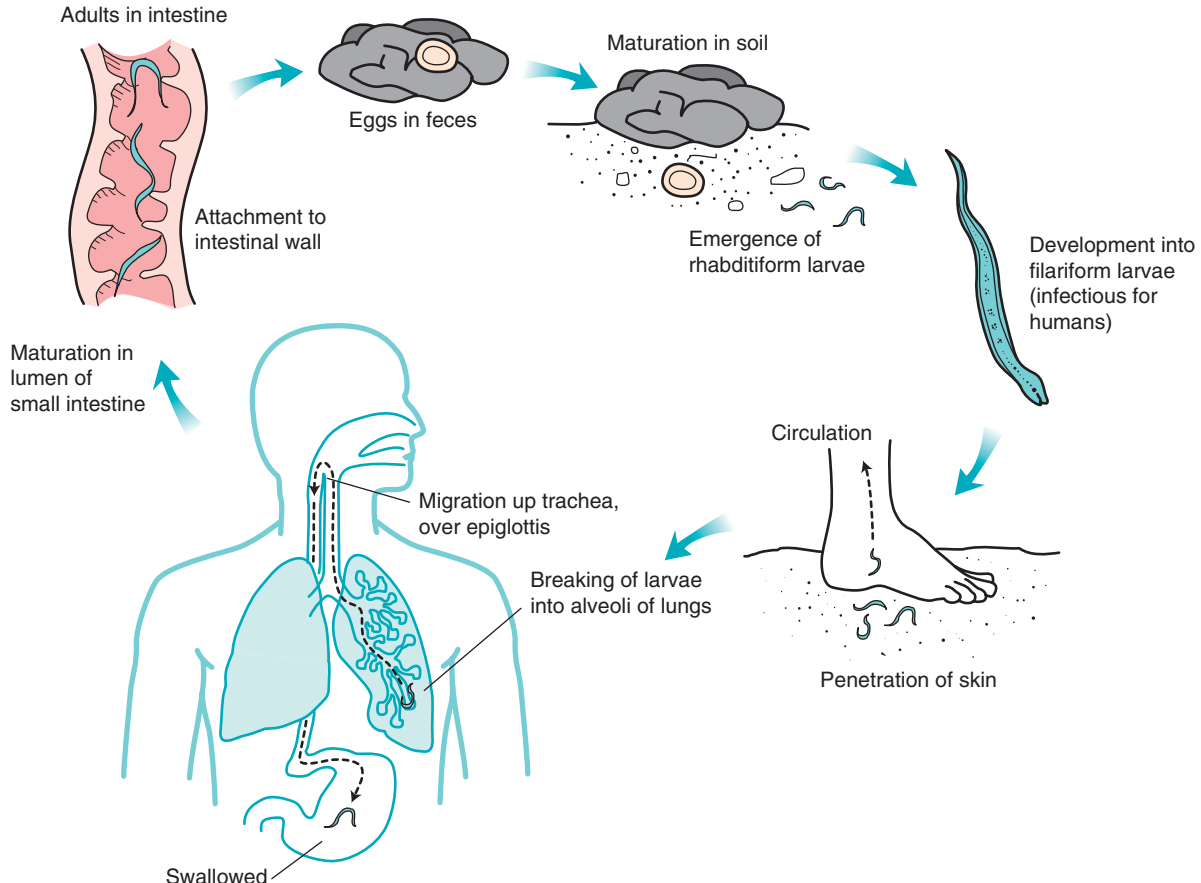


Fig. 28.69 Life cycle of the hookworm.

host becomes immunocompromised. In the United States, the prevalence rate ranges from 0.4% to 4%, with most cases found in people living in Appalachia, people living in rural areas of the Southeast, or immigrants from endemic areas.

Although many patients with *S. stercoralis* infection are asymptomatic, some may exhibit fever, nausea and vomiting, tracheal irritation, and sharp, stabbing pains that resemble those of an ulcer or other GI diseases, including pancreatitis. Chronic mild diarrhea may be present. The eosinophil count is often elevated—in some cases, up to 40%. Unlike hookworms, *S. stercoralis* larval penetration of skin does not cause a prominent papule, and migration through the lungs rarely elicits pneumonitis, but the patient may have wheezing and a mild cough.

In contrast with the mild symptoms in an immunocompetent host, patients with a drug-induced immunocompromised state (corticosteroids), lymphoma, human T-lymphotropic virus 1 infection, malignancy, or other conditions that cause T-cell depletion can develop severe infections, referred to as *disseminated strongyloidiasis* or *hyperinfection*. In this population, large numbers of the filariform larvae develop in the intestine in an autoinfective cycle and migrate from the intestine into the lungs and other organs, such as the liver, heart, and CNS, causing a fulminating, often fatal infection. Shortness of breath and coughing are common symptoms in

this group of patients. Fig. 28.72 is a Gram-stained smear of aspirated sputum showing coiled nematode larvae (arrows), the morphology of which is consistent with *S. stercoralis*.

Most infections in organ transplant recipients are caused by reactivation of latent or chronic infection, although some cases may result from primary infection. Individuals undergoing transplantation and who have a history of travel to or residence in endemic areas may need to be screened for *S. stercoralis*. Secondary bacterial infections that occur because of massive larval migration may be seen in up to 40% of patients with disseminated strongyloidiasis and can delay diagnosis of the underlying infection. The mortality rate in immunocompromised patients is over 80%; the usual causes of death are complications resulting in respiratory failure. Disseminated strongyloidiasis, however, is not common in patients with advanced AIDS, despite their immunocompromised status. Research into this seeming contradiction has shown that progression from intestinal to hyperinfection is dependent on an intact mucosal immune response that allows rhabditiform larvae to develop into infectious larvae in the gut leading to autoinfection. It is hypothesized that immune suppression in HIV prevents the development of the larvae in the gut, and autoinfection is halted.

The life cycle of *S. stercoralis* can take one of three phases (Fig. 28.73): (1) direct, which is similar to that of the

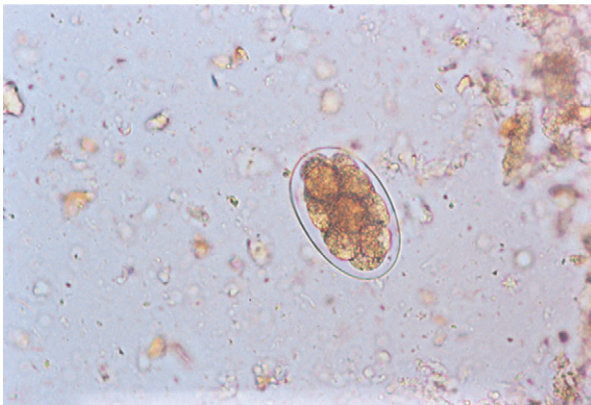


Fig. 28.70 Hookworm egg (unstained, ×400).

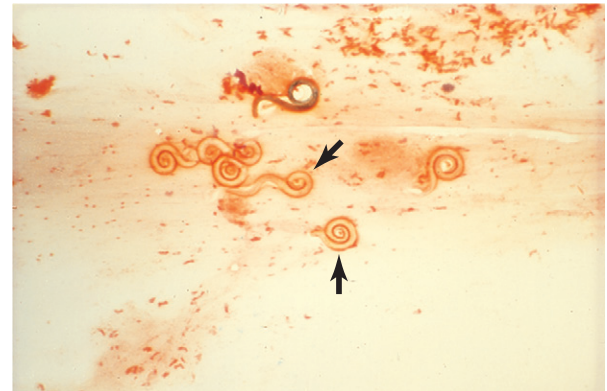


Fig. 28.72 Aspirated sputum, Gram stain, light microscopy, (×200). Coiled nematode larvae (arrows). Morphology consistent with *Strongyloides stercoralis*.



Fig. 28.71 A, Hookworm rhabditiform larva. Note the long buccal capsule and lack of prominent genital primordium (iodine wet mount, ×200). B, Hookworm rhabditiform larva, buccal capsule (iodine wet mount, ×200).

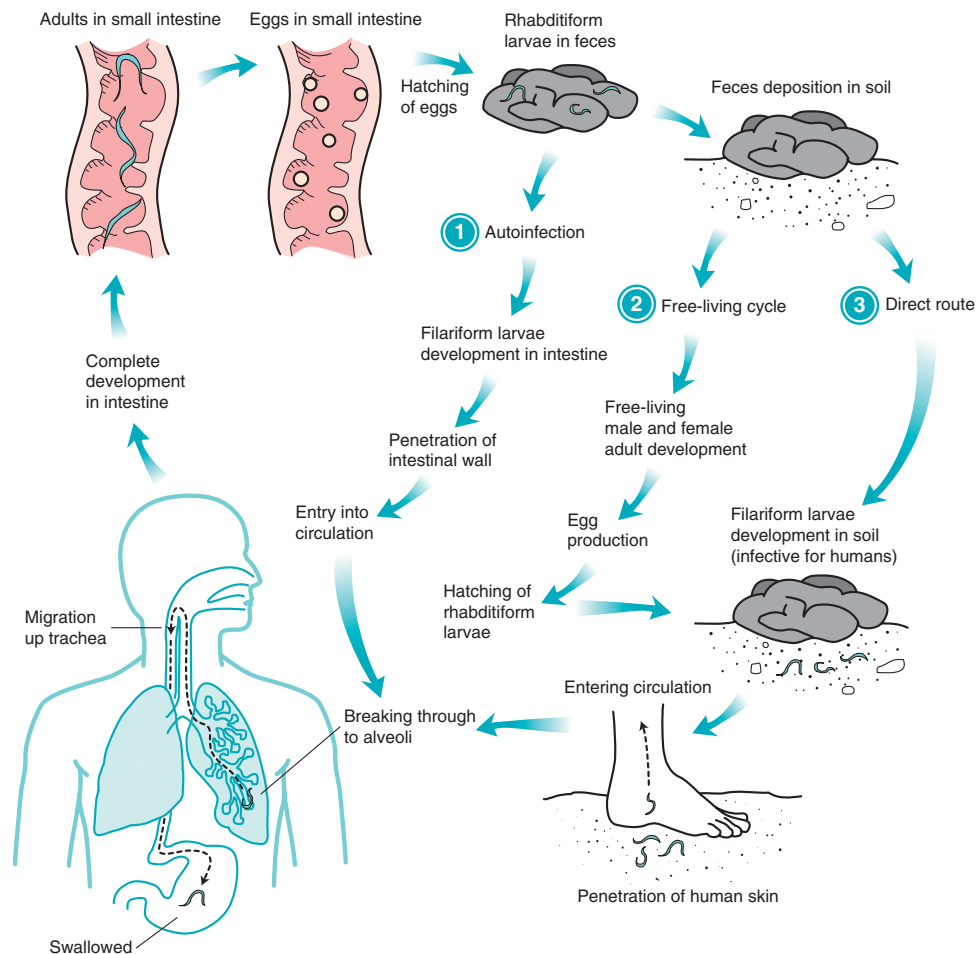


Fig. 28.73 Life cycle of *Strongyloides stercoralis*.

hookworm; (2) indirect, which involves a free-living phase; or (3) autoinfection. In the direct life cycle, the fertile egg hatches in the intestine and develops into the rhabditiform larva (noninfective form), which is passed in feces. In the soil, the rhabditiform larvae develop into filariform larvae, which are infective for humans by direct penetration. Once the larva has penetrated skin, it enters the blood circulation, breaks out from the capillaries in the lung, migrates up the bronchial tree and over the epiglottis, and enters the digestive tract, where it matures into the adult worm.

In the indirect life cycle, the rhabditiform larvae in the soil develop into free-living males and into females that produce eggs. At any point, the free-living cycle may revert and result in the production of infective filariform larvae. In most individuals, the autoinfective life cycle allows the initial infection to persist at low levels for years and is the underlying cause of hyperinfection. In this cycle, the rhabditiform larvae develop into the filariform larvae in the intestine rather than being passed in feces. These filariform larvae then penetrate the mucosa, enter the blood circulation, and return to the intestine to develop into adults.

The parasitic female threadworm is small (2.5 mm) and rarely seen in a stool specimen. No male has been identified in intestinal infections. The primary diagnostic stage in humans is the rhabditiform larva. It is 200 to 250 μm long, with a short buccal capsule (Fig. 28.74A), large bulb in the esophagus, and

prominent genital primordium located in its posterior half to posterior third (see Fig. 28.74B). The egg, which is rarely seen except in cases of severe diarrhea, resembles that of a hookworm. It is thin shelled, measures $54 \times 32 \mu\text{m}$, and often is segmented.

The filariform larva has a notched tail, is 500 μm long, and has an esophageal-to-intestinal ratio of 1:1. **Filariform larvae** may be identified in the sputum of patients with hyperinfection (Fig. 28.72). Fig. 28.75 shows a sheep blood agar plate inoculated with sputum, after 24-hour incubation with 5% carbon dioxide in air. Note heavy bacterial growth in the area of primary inoculation. The thin trails of colonies (arrows) lacking the surface of the agar are due to motile larvae. If clinical symptoms suggest *Strongyloides* infection but multiple stool specimens test negative for the larvae, a duodenal aspirate or biopsy specimen may be used for diagnosis because the organism lives in the upper small intestine.

There are no antigen-specific tests for *Strongyloides* infection, and diagnosis relies on identification of the larvae in stool. Antibody tests cannot distinguish current infection from past infection and are prone to yielding false-positive results in infections with other helminths. Ivermectin is the recommended therapy with albendazole used as an alternative. In hyperinfection or disseminated infections, it is recommended that immunosuppressive therapy be reduced if possible.



Fig. 28.74 **A**, *Strongyloides stercoralis* rhabditiform larva, buccal capsule (iodine wet mount, $\times 200$). **B**, *S. stercoralis* rhabditiform larva. Note the short buccal capsule and prominent genital primordium (unstained, $\times 200$).



Fig. 28.75 Sheep blood agar plate inoculated with expectorated sputum, 24-hour incubation with 5% carbon dioxide in air. Note heavy bacterial growth in the area of primary inoculation with thin trails of colonies (arrows) lacing the surface of the agar (see Fig. 28.72).

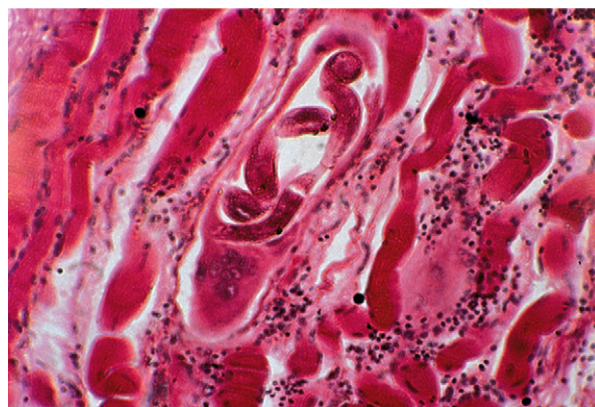


Fig. 28.76 *Trichinella spiralis* larva (biopsy specimen) (hematoxylin and eosin stain, $\times 200$).

Blood and tissue roundworm infections

Trichinella spiralis

Trichinosis is the infection of muscle tissue with the larval form of *T. spiralis*, a helminth whose adult stages live in the human intestine. Humans acquire the infection by eating undercooked meat, particularly pork that contains the larval forms. In recent years, ingestion of wild game has led to infection by other species of *Trichinella*. The larvae are released from the tissue capsule in the intestine and mature into adults. The female produces liveborn larvae that penetrate the intestinal wall, enter the circulation, and are carried throughout the body. Once the larvae enter the striated muscle, they begin a maturation cycle that is completed in about 1 month. The larvae coil and become encapsulated. Although larvae remain viable for many years, eventually the capsules calcify, and the larvae die.

During the intestinal phase, infected individuals have few symptoms, although diarrhea and abdominal discomfort may be present. Most symptoms occur during the migration and encapsulation periods; the severity of symptoms depends on the number of parasites, the tissues invaded, and the person's general health. Symptoms that occur during the larval phase are the result of an intense inflammatory response by the host.

Common symptoms include periorbital edema, fever, muscular pain or tenderness, headache, and general weakness. Muscle enzyme levels may be elevated. Splinter hemorrhages beneath the nails can be seen in many patients. A 40% to 80% increase in the number of eosinophils is common. Patients with symptoms should be treated with analgesics and general supportive measures. Steroids are given only in rare cases. The use of albendazole or mebendazole is effective when administered early in the infection, before the release of larvae. If diagnosis is delayed, the impact of antiparasitic drugs is less effective.

Because it is difficult to recover adults or larvae in a stool specimen, the diagnosis is often based on clinical symptoms and the patient's history. Biopsy of muscle tissue and identification of the encapsulated, coiled larva is the definitive diagnostic method. Fig. 28.76 shows a biopsy specimen of a muscle containing a larva of *T. spiralis*. Specimens from large muscles, such as the deltoid and gastrocnemius, should be stained for histologic examination (Fig. 28.77). The presence of calcified larvae on a radiographic film indicates infection. Serologic tests are available.

Larva migrans

Two forms of larva migrans exist in humans: cutaneous (creeping eruption) and visceral. In both cases, humans are the accidental host for nonhuman nematode larvae that are unable to

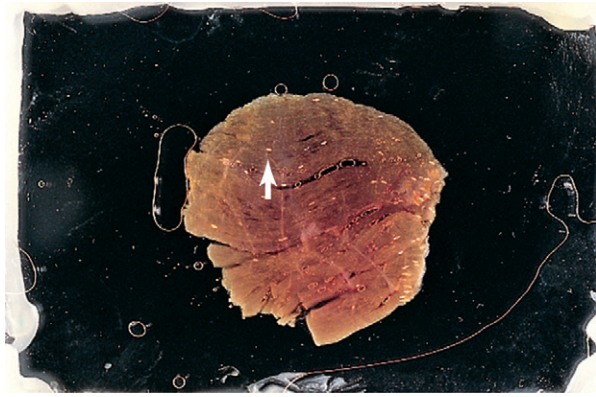


Fig. 28.77 Muscle tissue, directly viewed. Encysted calcified larvae (arrow). Morphology consistent with *Trichinella spiralis*.

complete their life cycle in humans. In the United States, cutaneous larva migrans occurs primarily in the Southwest, Mid-Atlantic, and Gulf Coast areas, and it is most commonly caused by the filariform larva of the dog or cat hookworm (*Ancylostoma braziliense*). The larva penetrates skin through a hair follicle, a break in skin, or unbroken skin. Once inside the body, it does not enter the circulation but wanders through the subcutaneous tissue, creating long, winding tunnels. Secretions from the larva create a severe allergic reaction, with intensely itchy skin lesions that are vesicular and erythematous. Secondary bacterial infections can result from scratching. The infection resolves within several weeks when the larva dies. Diagnosis is based primarily on history and clinical symptoms.

In visceral larva migrans, a human accidentally ingests the eggs of the dog roundworm (*Toxocara canis*) or cat roundworm (*Toxocara cati*). The larvae hatch in the intestine, penetrate the intestinal mucosa, and wander through the abdominal cavity and can enter the lung, eye, liver, or brain. The infection is seen primarily in children 1 to 4 years of age. Clinical symptoms include malaise, fever, pneumonitis, and hepatomegaly. An increase in the number of eosinophils ranging from 30% to 50% and as high as 85% has been reported. CNS complications may develop. Eye invasion is referred to as *ocular larva migrans*, and in these cases, eosinophilia is usually absent. The diagnosis is made on the basis of clinical findings and results of serologic tests using *Toxocara*-specific antigens.

Filarial worms

Filarial worms are roundworms of blood and tissue found primarily in tropical areas of the world. At least eight species infect humans. Those considered most pathogenic are *Brugia malayi*, *Wuchereria bancrofti*, *Onchocerca volvulus*, and *Loa loa*. Lymphatic filariasis (caused by *W. bancrofti*, *B. malayi*, and *Brugia timori*) is the second most common mosquito-borne disease, after malaria. In addition, the nonpathogens *Mansonella ozzardi*, *Mansonella perstans*, and *Mansonella streptocerca* may be seen in clinical specimens. *W. bancrofti* is the most common species and has the greatest geographic distribution being found throughout the tropics and subtropics.

The adult filarial worms give birth to liveborn larvae referred to as *microfilariae*. Identification of the various species depends on the morphology of the microfilaria, periodicity (microfilaria migration), and location of the worms in

the host. Important microfilaria morphologic characteristics include the presence or absence of a sheath (the remnant of the egg from which the larva hatched) and the presence and arrangement of nuclei in the tail. Table 28.10 compares the species of microfilariae commonly found in humans.

Adult worms, which may range in size from 2 to 50 cm, live in human lymphatics, muscles, or connective tissues. Mature females produce microfilariae that are the infective stage for the insect during the insect's blood meal. Once ingested, microfilariae penetrate the insect's gut wall and develop into infective third-stage (filariform) larvae. These larvae enter the insect proboscis and are introduced into human circulation when the insect feeds. Fig. 28.78 illustrates a generalized life cycle for microfilariae.

Wuchereria bancrofti

W. bancrofti is the causative agent of bancroftian filariasis and elephantiasis. Several genera/species of mosquito serve as the insect vector. The adult filarial worm lives in the lymphatics and lymph nodes, especially those in the lower extremities. The presence of the adults initiates an immunologic response consisting of cellular reactions, edema, and hyperplasia. A strong granulomatous reaction with production of fibrous tissue around dead worms ensues. The resulting reaction causes the small lymphatics to become narrowed or closed, causing increased hydrostatic pressure, with subsequent leakage of fluid into the surrounding tissue. During this period, the patient may experience generalized symptoms, such as fever, headache, and chills, as well as localized swelling, redness, and lymphangitis, primarily at sites in the male and female genitalia and extremities. Elephantiasis, a debilitating and deforming complication, occurs in less than 10% of infections, usually after many years of continual filarial infection. Chronic obstruction to the lymphatic flow results in lymphatic varices, fibrosis, and proliferation of dermal and connective tissue. The enlarged areas eventually develop a hard, leathery appearance.

Diagnosis of *W. bancrofti* should include the examination of a blood specimen obtained at night (10 PM to 2 AM) for the presence of microfilariae. Blood specimens may be examined immediately for live microfilariae or may be pooled on a slide and stained. Filtration of up to 5 mL of blood through a 5- μ m nucleopore filter can detect light infections. The microfilariae of *W. bancrofti* are sheathed, and the nuclei do not extend to the tip of the tail (Fig. 28.79). Three antigen detection assays are available, but none have U.S. FDA approval. No antibody detection tests are available.

Brugia malayi

B. malayi, another nocturnal microfilarial species, is limited to the Far East, including Korea, China, and the Philippines. Mosquitoes of the genera *Mansonia* and *Aedes* have been shown to transmit the organism. The pathologic aspects of the disease and the clinical symptoms are the same as those seen with *W. bancrofti* infections. The distinguishing characteristics of the microfilariae are the presence of a sheath and the arrangement of tail nuclei—the nuclei extend to the tip, but a space separates the two terminal nuclei.

Loa loa

Infection with *L. loa*, the eye worm, is limited to the African equatorial rainforest, where the fly vector (*Chrysops*) breeds. Adult worms, which may live for as long as 15 years in

Table 28.10 Comparisons of microfilariae

Organism	Arthropod vector	Periodicity	Location of adult, microfilaria	Tail morphology
<i>Wuchereria bancrofti</i>	Mosquito (<i>Culex</i> , <i>Aedes</i> , <i>Anopheles</i> spp. and others)	Nocturnal	Lymphatics, blood	Sheathed Nuclei do not extend to tip of tail
<i>Brugia malayi</i>	Mosquito (<i>Aedes</i> , <i>Mansonia</i> spp.)	Nocturnal	Lymphatics, blood	Sheathed Terminal nuclei separated
<i>Loa loa</i>	Fly (<i>Chrysops</i> sp.)	Diurnal	Subcutaneous tissue, blood	Sheathed Nuclei extend to tip of tail
<i>Onchocerca volvulus</i>	Fly (<i>Simulium</i> sp.)	Nonperiodic	Subcutaneous nodule, subcutaneous tissue	Unsheathed Nuclei do not extend to tip of tail
<i>Mansonella ozzardi</i>	Midge (<i>Culicoides</i> sp.)	Nonperiodic	Body cavity, blood, skin	Unsheathed Nuclei do not extend to tip of tail
<i>Mansonella perstans</i>	Midge (<i>Culicoides</i> sp.)	Nonperiodic	Mesentery, blood	Unsheathed Nuclei extend to blunt tip of tail
<i>Mansonella streptocerca</i>	Midge (<i>Culicoides</i> sp.)	Nonperiodic	Subcutaneous, skin	Unsheathed Nuclei extend to tip of hooked tail

humans, migrate through the subcutaneous tissue, causing temporary inflammatory reactions called *Calabar swellings*. These characteristic swellings can cause pain and pruritus that last about 1 week before disappearing, only to reappear in another part of the body. The adult worm can often be seen as it migrates across the surface of the eye. Diagnosis can be based on the presence of Calabar swellings or of the adult worm in the conjunctiva of the eye. Microfilariae may be seen in a blood specimen if it is taken during the day, especially around noon, when migration peaks. The microfilaria is sheathed, and nuclei extend to the tip of the tail.

Onchocerca volvulus

Infection with *O. volvulus* is referred to as *onchocercosis*, or *river blindness*. The organism can be found in Africa and South and Central America; transmission occurs by the bite of the black fly (*Simulium*). Adult worms are encapsulated in fibrous tumors in the subcutaneous tissues of humans. Microfilariae can be isolated from the subcutaneous tissue, skin, and the nodule itself, but are rarely found in blood or lymphatic fluid. The nodules in which adults live may measure up to 25 mm and can be found on most parts of the body. They are the result of an inflammatory and granulomatous reaction around the adult worms. Fig. 28.80 depicts a cross-section of

tissue containing these organisms. Blindness, the most serious complication, results when microfilariae collect in the cornea and iris, causing hemorrhage, keratitis, and atrophy of the iris. The presence of endosymbiotic bacteria of the genus *Wolbachia* has been linked to stimulation of the host immune response and may contribute to the inflammatory tissue reaction.

Diagnosis involves clinical symptoms, such as the presence of nodules, and microscopic identification of microfilariae. The diagnostic method used is the *skin snip*, in which a small slice of skin is obtained and placed on a saline mount. Microfilariae with no sheath and with nuclei that do not extend into the tip of the tail are characteristic of this organism. Because the skin snip is painful and poses a risk of infection, researchers are trying to develop alternate diagnostic methods. NAATs are being used in research laboratories. In addition, serologic assays using recombinant antigens have demonstrated high sensitivity and specificity.

Mansonella spp.

M. ozzardi, *M. streptocerca*, and *M. perstans* are filarial worms not usually associated with serious infections. They are transmitted by midges belonging to the genus *Culicoides*. The microfilariae of *M. streptocerca* are found in skin.

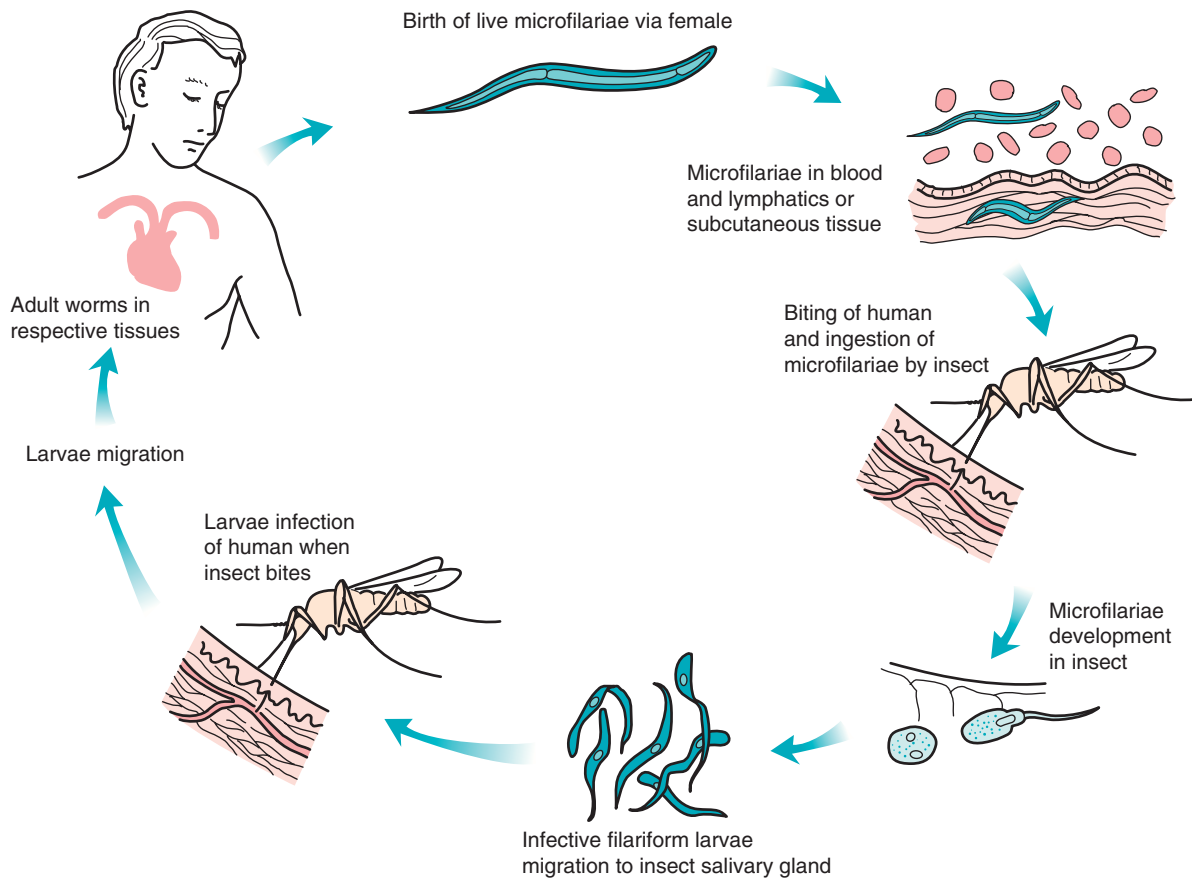


Fig. 28.78 Generalized life cycle of microfilariae.

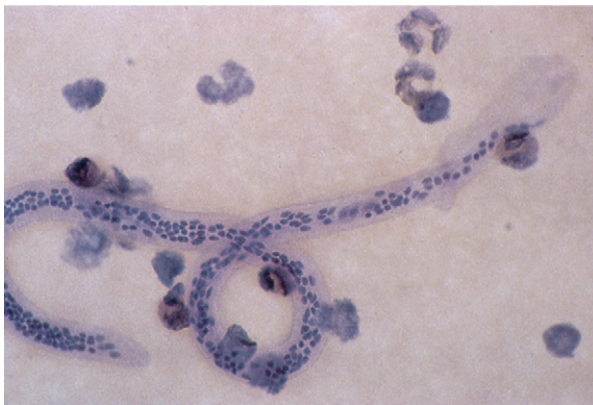


Fig. 28.79 *Wuchereria bancrofti* microfilaria. Note the faintly staining sheath extend from both ends of organism (Giemsa stain, $\times 1000$).

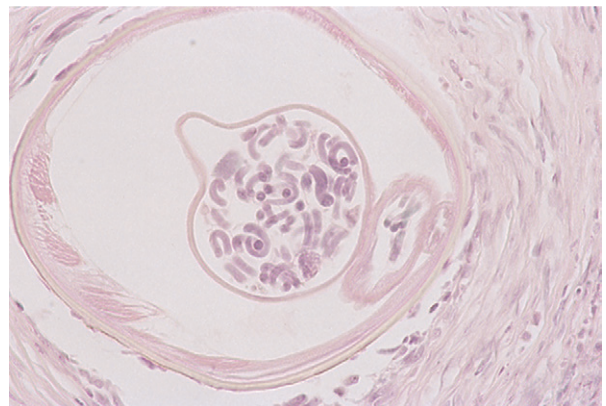


Fig. 28.80 Cross-section of tissue infected with *Onchocerca volvulus* (hematoxylin and eosin stain, $\times 100$).

They are unsheathed and have nuclei that extend to the end of the so-called *shepherd's crook tail*. Microfilariae of *M. ozzardi* and *M. perstans* are found in blood as unsheathed organisms. *M. ozzardi* microfilariae have tails with nuclei that do not extend to the tips, whereas the nuclei in the tail of an *M. perstans* microfilaria extend to the tip.

Dracunculus medinensis

D. medinensis, known as the *guinea worm* (also called the *fiery serpent* of the Israelites described in the Bible), historically

caused serious infections in the Middle East, parts of Africa, and India. It is often found in areas where step-down wells are used. Several health agencies, including the WHO and the United Nations Children's Fund, launched an eradication program in 1986. At that time, an estimated 3.5 million people were infected. By 2012, only 542 cases were reported, and dracunculiasis was limited to four African countries. In 2020, only 27 cases were reported, raising hopes that the disease may be totally eradicated.

Adult worms mature in the deep connective tissue, and the gravid females migrate to the subcutaneous tissue. Initially, a

painful, blisterlike, inflammatory papule appears on the leg in the area where the gravid female is present. The papule ulcerates, and when the person's body comes in contact with water, the female worm exposes her uterus through the ulceration and releases larvae into the water. Patients may experience nausea and vomiting, urticaria, and dyspnea before the rupture of the worm's uterus. If the worm is broken during an attempt to remove it, the patient may experience a severe inflammatory reaction and secondary bacterial infection.

Humans acquire the infection by ingesting a copepod (*cyclops*) that contains an infective larva. The larva is released in the intestine, penetrates the intestinal wall, and migrates to the body cavity, in which males and females mature. When mature and gravid, the female migrates through the subcutaneous tissue to the arm or leg to release liveborn larvae into the water. The rhabditiform larvae are then ingested by the copepod.

The diagnosis is made from the typical appearance of the lesion. Although organism-specific antibodies develop with infection, immunity does not develop, and an individual can be infected multiple times. Treatment is based on the extraction of the adult worm. The worm can be wound around a stick and eased out a few centimeters each day. Analgesics and topical agents, such as metronidazole or thiabendazole, can help in the removal process, although they do not kill the adult worm. Because this method can rupture the worm, surgical excision is preferred.

POINTS TO REMEMBER

- Protozoan cysts, helminth eggs, and helminth larvae can be identified on a wet mount preparation of a fecal concentrate. The identification of protozoan trophozoites and cysts is confirmed on a permanently stained smear.
- Rapid diagnostic tests based on antigen detection (e.g., immunochromatographic) are routinely used for detection of *Giardia duodenalis*, *Cryptosporidium* spp., and *Entamoeba histolytica*.
- Multiplex nucleic acid assays for intestinal pathogens include several organisms—usually including the parasites *G. intestinalis*, *Cryptosporidium* spp., and *E. histolytica*.
- Fecal-oral transmission is the route of infection for enteric protozoa. The cyst is the infective stage, and the trophozoite is the stage that divides and causes tissue damage.
- The major intestinal protozoan pathogens include *E. histolytica* and *G. duodenalis*. *D. fragilis* and *B. hominis* cause symptomatic infections in some patients. There are several commensal protozoa that must be differentiated from the pathogens to ensure appropriate therapy when necessary.
- *E. histolytica*, *E. dispar*, *E. moshkovskii*, and *E. bangladeshi* are morphologically identical, and specific immunoassay techniques must be performed to differentiate the pathogenic *E. histolytica* from the other three nonpathogenic species. If ingested RBCs are present in the trophozoite, the organism can be reported as *E. histolytica*.
- *N. fowleri*, *Acanthamoeba* spp., and *B. mandrillaris* are free-living amebae that can infect humans. *N. fowleri* causes PAM, which is rapidly fatal. *Acanthamoeba* spp. cause keratitis, skin infections, or granulomatous amebic encephalitis, whereas *B. mandrillaris* is associated with GAE and skin infections.
- The genera *Leishmania* and *Trypanosoma* are blood flagellates of humans transmitted by insect bites.
- Human malaria can be caused by five different *Plasmodium* spp.: *P. vivax*, *P. ovale*, *P. malariae*, *P. falciparum*, and *P. knowlesi*. *P. vivax* is the most prevalent.
- The life cycle of malaria is complex, with asexual reproduction taking place in human RBCs (intermediate host) and sexual reproduction occurring in the gut of the mosquito (definitive host).
- Identification of *Plasmodium* spp. is made by observing characteristics of the infected RBCs and the malarial organism on a Wright- or Giemsa-stained peripheral blood smear.
- One rapid diagnostic test (*P. vivax* and *P. falciparum*) is approved for use in the United States for diagnosing malaria. Microscopic confirmation is still performed.
- *B. microti*, an intraerythrocytic parasite, morphologically resembles *P. falciparum* on blood smears.
- The intestinal Apicomplexa species includes *C. parvum*, *C. belli*, and *C. cayetanensis*; the infective stage for humans is the acid-fast–positive oocyst.
- The microsporidia are small intracellular organisms that can infect the gastrointestinal tract and other tissue. Even though they are classified as fungi, the microsporidia are often discussed with eucaryotic parasites because of their morphology and pathogenesis.
- *T. gondii* causes a tissue infection that is usually asymptomatic in immunocompetent hosts. In patients with AIDS, a latent infection can reactivate and cause encephalitis or pneumonia. Congenital transmission can result in serious complications.
- For most flukes, diagnosis is made by finding the egg in a fecal specimen. In the case of the lung fluke (*P. westermani*), the egg may be found in sputum; in the case of *S. haematobium*, the egg is found in urine.
- Humans are the definitive hosts, and animals or insects serve as intermediate hosts for most tapeworms infecting humans.
- Eggs of the beef tapeworm, *T. saginata*, and eggs of the pork tapeworm, *T. solium*, are identical and must be reported as *Taenia* sp.
- The diagnostic stage for a roundworm may be an egg or a larval form, depending on the species.
- Pinworm (*E. vermicularis*) infection is common in children and is diagnosed by finding eggs on a cellophane tape preparation or pinworm paddle. Eggs are laid on the perianal area when the female migrates out through the anus at night.
- Human hookworm infections are generally caused by *N. americanus* and *A. duodenale*. Recently, a third species (*A. ceyanicum*) pathogenic for humans was identified. The eggs are identical and are reported as “hookworm eggs.”
- Eggs of *S. stercoralis* are not usually present in the stool; the typical diagnostic stage is the rhabditiform larva.
- *T. spiralis* is acquired when humans ingest raw or undercooked pork containing the larval form. Diagnosis of trichinosis is made through biopsy of tissue to identify the coiled larval stage.
- Diagnosis of filarial worm infection is made by observing the microfilariae in blood or tissue specimens. Larval characteristics include the presence or absence of a sheath and the location and arrangement of nuclei in the tail of the microfilariae.

LEARNING ASSESSMENT QUESTIONS

- A trichrome-stained smear of a patient's fecal specimen shows the presence of cysts that are oval and approximately 11 μm in size and have four nuclei containing large karyosomes with no peripheral chromatin and a cluttered appearance in the cytoplasm. What is the most likely identification of the organism? Is the organism considered a pathogen?
- A patient with a history of travel to Africa has fever and chills. The physician suspects malaria and orders a blood smear for examination. Why should you do both a thin film and a thick film? Why would final species identification be made from the thin smear?
- Give the major characteristics (including size) that you would use to identify eggs of the following organisms: *Taenia* spp., *Ascaris lumbricoides*, *Trichuris trichiura*, and hookworm.
- Describe the diagnostic method you would use to detect *Enterobius vermicularis* eggs that would not be used with the other types of eggs of intestinal helminths. Explain your answer.
- Describe the microscopic characteristics you would use to differentiate the oocysts of *Cyclospora cayetanensis* and *Cryptosporidium parvum*. Include size, appearance on routine wet mount or trichrome stain, and appearance with special stains.
- You identify a trophozoite that is approximately 22 μm in diameter on a trichrome-stained smear of a stool sample. There is a single nucleus that shows even peripheral chromatin and a small central karyosome. The cytoplasm is relatively clean, but ingested red blood cells are seen. What is the most likely identification of the organism? Is the organism considered a pathogen? If yes, describe the typical patient symptoms and possible complications.
- For both *Cryptosporidium* spp. and *Strongyloides stercoralis*, explain the mechanism of autoinfection in the life cycle and why this phase contributes to increased severity of infection.
- You are examining a blood smear and find an extracellular structure that is approximately 18 μm long. It is tapered at both ends and has an anterior flagellum. An undulating membrane extends the length of the body. What is the genus of this organism? What is the morphologic stage of this organism? With which two diseases do you see this stage in blood?
- Compare primary amebic meningoencephalitis and granulomatous amebic encephalitis. Include the following in your discussion: causative organism, population usually infected, route of infection, clinical symptoms, and method of diagnosis.
- For *Toxoplasma gondii*, *Cryptosporidium* spp., and *S. stercoralis*, compare the clinical presentation in the immunocompetent host and in the immunocompromised host.
- Which of the following are remnants of eosinophils in the stool of some patients with an intestinal parasite infection?
 - Ameboma
 - Charcot-Leyden crystals
 - Morulae
 - Weil-Felix granules
- An international male student from Egypt comes to the campus clinic complaining of blood in his urine. A urinalysis reveals an elongated structure that measures about 110 to 170 μm $\times$ 40 to 70 μm in size and has a terminal spine. Which of the following should be suspected?
 - Schistosoma japonicum* egg
 - Trichuris trichiura* egg
 - Schistosoma haematobium* egg
 - Enterobius vermicularis* egg
- A patient with recent history of camping has explosive, foul-smelling, nonbloody diarrhea. An ova and parasite examination of the stool shows oval structures, about 12 $\times$ 8 μm , with four oval nuclei, each with a large central karyosome, midline axonemes, and two median bodies. Which of the following matches this description?
 - Giardia duodenalis* cyst
 - Entamoeba histolytica* cyst
 - Cryptosporidium parvum* oocyst
 - Chilomastix mesnili* cyst
- Several children at a daycare center are having trouble sleeping and are showing increasing signs of restlessness. One child was recently diagnosed with an intestinal parasite, so the parents are informed that other children should be tested. The parasite egg found during examination is described as embryonated, containing a C-shaped larva, oval, colorless, and slightly flattened on one side. It measures approximately 50 to 60 μm $\times$ 20 to 30 μm . Which of the following matches this description?
 - Enterobius vermicularis*
 - Hookworm
 - Ascaris lumbricoides*
 - Trichuris trichiura*
- Although uncommon, which intestinal parasite is known to leave the colon and cause metastatic diseases, especially liver abscesses?
 - Giardia lamblia*
 - Entamoeba histolytica*
 - Cryptosporidium parvum*
 - Microsporidia*
- What is the most commonly identified intestinal protozoan parasite in the United States?
 - Cryptosporidium parvum*
 - Entamoeba histolytica*
 - Giardia duodenalis*
 - Microsporidia*
- This pathogen can be seen in the sputum and bronchoalveolar fluid of patients with a hyperinfestation syndrome. What is the name of this pathogen?
 - Ascaris lumbricoides*
 - Entamoeba histolytica*
 - Giardia duodenalis*
 - Strongyloides stercoralis*
- Which parasite is responsible for causing a form of blindness in West Africa?
 - Whipworm
 - Hookworm
 - Onchocerca volvulus*
 - Wuchereria bancrofti*

19. A corneal scraping from a patient with keratitis is submitted to the laboratory for examination. Which parasite would you look for in this type of clinical sample?
 - a. *Naegleria* sp. trophozoites
 - b. *Acanthamoeba* sp. cysts and trophozoites
 - c. *Trichinella spiralis* larvae
 - d. *Toxoplasma gondii* oocysts
20. While examining a peripheral blood smear stained with Giemsa-Wright stain, the hematologist notices several red blood cells that contain delicate malarial ring-forms. Further examination shows gametocytes that are banana- or crescent-shaped. Which *Plasmodium* sp. demonstrates these characteristics?
 - a. *Plasmodium vivax*
 - b. *Plasmodium malariae*
 - c. *Plasmodium falciparum*
 - d. *Plasmodium ovale*

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Clinical virology

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CHAPTER OUTLINE

Characteristics of viruses, 710

Structure, 710
Taxonomy, 710
Viral replication, 710

Laboratory diagnosis of viral infections, 713

Specimen selection, collection, and transport, 713
Appropriate specimens for maximum recovery, 713
Methods in diagnostic virology, 714

Double-stranded DNA viruses, 718

Adenoviridae, 718
Herpesviridae, 719
Papillomaviridae, 726
Poxviridae, 726

Single-stranded DNA viruses, 727

Parvoviridae, 727

Double-stranded RNA viruses, 728

Reoviridae, 728

Single-stranded RNA viruses, 729

Arenaviridae, 729
Bunyavirales, 729
Caliciviridae, 730
Coronaviridae, 731
Filoviridae, 732
Flaviviridae, 733
Orthomyxoviridae, 734
Paramyxoviridae, 736
Picornaviridae, 738
Retroviridae, 739
Rhabdoviridae, 742
Togaviridae, 742

Hepatitis viruses, 743

Hepatitis A virus, 744
Hepatitis B virus, 744
Hepatitis D virus, 745

Hepatitis C virus, 747
Hepatitis E virus, 748
Other hepatitis viruses, 748

Prions, 749

Treatment and management of viral infections, 749

Antiviral therapy, 749
Vaccines, 749

Bibliography, 751

OBJECTIVES

After reading and studying this chapter, you should be able to:

1. Describe the characteristics of viruses and how they differ from bacteria.
2. Describe how viruses replicate.
3. Describe the proper procedures for collection and transport of specimens for viral detection.
4. Name the appropriate specimen for maximum recovery of the suspected viral agent.
5. Compare the different methods used in the diagnosis of viral infections.
6. Explain the advantages and limitations of cell cultures for diagnosing viral infections.
7. Explain the advantages and limitations of rapid viral antigen detection methods.
8. Discuss the indications and limitations of serologic assays in the diagnosis of viral infections.
9. Describe how *cytopathic effect* is used to presumptively identify viral agents.
10. Evaluate the vaccination program for influenza.
11. List common opportunistic infections and other indicators of acquired immunodeficiency syndrome.
12. Create an algorithm for the serologic diagnosis of human immunodeficiency virus infection.
13. Compare the genomes and modes of transmission of the human hepatitis viruses.
14. Develop an algorithm for the serologic diagnosis of viral hepatitis.

15. Interpret the results of a hepatitis serologic profile.
16. For each of the viral agents presented in this chapter, discuss how the virus is transmitted, how the infection is produced, and the most effective method of laboratory diagnosis.
17. Discuss common treatment and prevention strategies for viral infections.

KEY TERMS

Aneuploid	Koplik spots
Antigenic drift	Nucleocapsid
Antigenic shift	Nucleoside reverse transcriptase inhibitors (NRTIs)
Arboviruses	Obligate intracellular parasites
Capsid	Pandemic
Cell cultures	Primary cell cultures
Continuous cell cultures	Prions
Cytopathic effect	Syncytia
Diploid	Tissue culture
Envelope	Vaccinia virus
Hemagglutinin	Virion
Heteroploid	

Case in point

On March 2, 2020, a 65-year-old male patient with a 14-year history of chronic lymphocytic leukemia along with hypogammaglobulinemia, chronic leukocytosis, and severe anemia presented to the emergency department of a local community hospital with lower back, lower hip, and lower extremity pain. He underwent surgery for a hip fracture related to his cancer on March 5, 2020, and was subsequently transferred to a rehabilitation facility. He was readmitted to the hospital for anemia 2 weeks later. A chest x-ray was normal, and other tests were unremarkable. A chest computed tomography (CT) was also unremarkable. The patient had no respiratory or systemic symptoms. He could not return to the rehabilitation center because of a confirmed outbreak of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection, the cause of coronavirus disease 2019 (COVID-19), at the facility. Because he lived in the rehabilitation facility around the time of the COVID-19 outbreak, he was tested and found positive for SARS-CoV-2 on March 25, 2020.

After the SARS-CoV-2 diagnosis, the patient was kept in an isolation unit in a single room with negative air pressure. Because of acquired hypogammaglobulinemia caused by his cancer, the patient received intravenous (IV) immunoglobulin every 4 weeks as part of his treatment regimen. In early May, he was transfused with 200 mL of SARS-CoV-2 convalescent plasma under a U.S. Food and Drug Administration (FDA) emergency investigational new drug protocol. The infection persisted, and 1 week later, he received another 200-mL dose of convalescent plasma from a different donor. Infectious SARS-CoV-2 was successfully cultured from the nasopharyngeal swabs collected on day 35 and day 72. For the next 18 weeks, he was tested for SARS-CoV-2 another 20 times using an in-house real-time reverse-transcription polymerase chain reaction (RT-PCR) test and remained positive through July 16, 2020 (113 days after the initial positive RT-PCR test). Subsequently, he tested negative on six consecutive swabs from July 22 to August 10, indicating that the infection had finally cleared. While the patient remained asymptomatic for the entire duration of his hospitalization, culturable virus was obtained on day 25, day 60, and day 80 of his admission.

Issues to consider

After reading the patient's case history, consider:

- How the patient's medical history affected his viral infection
- What information is obtained from the laboratory, radiograph, and CT results
- Could this patient have been returned to the rehabilitation facility sooner? If yes, why? If no, why not?
- Other testing for SARS-CoV-2 includes antigen testing and testing for immunoglobulin G (IgG) and IgM antibodies. Would the patient's diagnosis or treatment have improved with any of these assays?

Clinical virology continues to be a challenging and exciting area of clinical microbiology. It has evolved over the years from viral diagnostic testing performed in only very few, highly specialized laboratories to being available in many modern laboratories of today. Most of the older, traditional diagnostic methods were time-consuming, cumbersome, and required significant expertise because they were primarily based on cell culture, serology, and microscopy (both bright-field and electron). Often, results were too slow to be clinically useful and were perhaps even irrelevant to the clinician.

With the recent emergence of molecular diagnostic testing for viral infections, detection is much faster, more sensitive, and more specific, resulting in earlier intervention, quicker treatment, and more favorable patient outcomes. The development of increasingly larger molecular panels to detect viral infections from blood, cerebrospinal fluid (CSF), and respiratory samples has made the diagnosis of these infections more clinically useful. Molecular technology is becoming more cost-effective and more common in the routine clinical microbiology laboratory.

Viruses have been ominous clinical threats since documented medical history. Viral infections that begin in local communities as endemic disease could eventually spread globally, leading to pandemics (as we are currently facing with COVID-19) that burst into our daily lives, disrupting all facets of life. The most deadly viral pandemic of the last century was caused by influenza A virus (H1N1) in 1918. This disease, known as the Spanish flu, resulted in at least 500 million infections and 50 million deaths worldwide. There have been several deadly viral pandemics in the last 40 years, including the following:

- The human immunodeficiency virus (HIV), identified as the causative agent of acquired immunodeficiency syndrome (AIDS), was first identified in 1981 and is still causing an ongoing epidemic in many parts of the world. HIV has infected over 79 million people and resulted in 36 million deaths. The development of antiretroviral therapy (ART) has helped to decrease mortality and improve the quality of life of infected individuals.
- The West Nile virus (WNV) was introduced into North America in 1999 and within a few years became endemic to most regions of the United States. In 2012 there was a resurgence, resulting in double the yearly cases seen before that time.
- Dengue virus, the causative agent of dengue fever (DF), is one of the most concerning members of the family Flaviviridae. Spread by mosquito bites, it is emerging as a pandemic in many countries. More than 1 billion people are at risk of dengue infection worldwide.

Brazil has been plagued with outbreaks of DF for over 30 years, and currently over 1 million cases are reported annually.

- The first coronavirus pandemic was caused by severe acute respiratory syndrome coronavirus 1 (SARS-CoV-1). The infections began in late 2002 and spread rapidly worldwide in 2003. Although the mortality rate was high, the global outbreak was quickly contained.
- In 2007, a Zika virus outbreak was reported on the island of Yap (Micronesia), where nearly 75% of the population was infected. In Brazil, a Zika virus outbreak started in November 2015 and continued into 2016 with subsequent spread to the Americas, including the southern United States. In this outbreak, there was a link to microcephaly in neonates.
- Influenza A virus subtype H1N1 (2009) first emerged in Mexico and rapidly spread to other countries, resulting in thousands of deaths worldwide. Since then, several variants of influenza virus subtypes appeared epidemically. One variant, influenza A (H3N2), affected humans and swine throughout the United States and was implicated in infections in visitors to county fairs. The key to control the spread of influenza viruses is to develop new vaccines based on the subtypes identified in the population every year. Annual flu vaccines have been successful in preventing flu pandemic outbreaks.
- Middle East respiratory syndrome coronavirus (MERS-CoV) emerged in Saudi Arabia in 2012 and later spread to 27 countries, resulting in about 2000 cases and 866 deaths.
- The spread of chikungunya virus to Caribbean countries and territories of the United States was first seen in late 2013 and reported in Florida, Puerto Rico, and the U.S. Virgin Islands. Prior to 2013, viral outbreaks were limited to countries in Africa, Asia, Europe, and the Indian and Pacific Oceans.
- In West Africa, an Ebola outbreak started in 2014 and lasted well into 2016. Over 28,000 cases in 10 countries were reported. During the outbreak, 11 patients were treated in the United States. Two cases were travel associated, and two cases involved health care workers caring for one of the patients. An additional 7 patients were transported by chartered aircraft to hospitals for treatment. Additional outbreaks were reported in 2017, 2018, 2020, and 2021.
- SARS-CoV-2 was first identified in China in late 2019 as cases of atypical pneumonia. The infection spread rapidly across the world, leading to global shutdowns and travel restrictions. As of July 2022, the World Health Organization (WHO) reported 544,324,069 confirmed cases of COVID-19, including 6,332,963 deaths (and still counting). The rapid development of effective mRNA-based vaccine against the virus helped to bring a state of some normalcy and decline in mortality, but the virus is still mutating rapidly and the pandemic is far from over.
- In 2022, monkeypox caused outbreaks in several nonendemic countries, including the United States. Sporadic outbreaks of monkeypox virus have been reported in many African countries since 2003. The first outbreak of monkeypox in the United States occurred in 2003. Monkeypox virus is closely related to smallpox virus, and the smallpox vaccine has been effective in preventing monkeypox infections.

Viruses continue to thrive in a small subset of hosts (human and sometimes animal reservoirs) even after a pandemic ends. For example, HIV continues to devastate entire continents, effectively reducing large portions of each generation. Mosquitoes continue to spread dengue virus throughout the world with significant impact. Over the years, there has been a rise in infections by enterovirus 71 (EV71), which has killed hundreds of children throughout parts of the Asian continent. Every year about 5% to 15% of the world's population is infected with influenza virus. Despite influenza surveillance programs, reliable annual vaccines, and dependable antiviral medications, several of these infected individuals develop severe disease. Influenza virus and other viruses lead to hundreds of thousands of deaths globally. This chapter discusses basic virology, including the advances and challenges in clinical virology in the modern clinical laboratory and how the laboratory helps diagnose viral illnesses.

Characteristics of viruses

Structure

All viruses contain nucleic acid and a protein **capsid**, with some viruses having up to 90% of their mass from the protein component. Typically, viruses contain a viral genome of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA). The genome can be double-stranded (ds) or single-stranded (ss). The genome and its protein coat together are referred to as the **nucleocapsid**. The entire virus particle is called the **virion**. Some viruses also have a phospholipid, labile **envelope** surrounding the virion. Enveloped viruses are usually more susceptible to inactivation by high temperature, extreme pH, and chemicals compared with non-enveloped (or naked) viruses. The envelopes are of host origin, derived from the plasma membrane, but contain virus-encoded proteins.

The morphology of virions is helical, polyhedral (e.g., *icosahedral*, a geometric shape with 20 triangular sides), or complex. The envelope masks the shape of the virion, so most enveloped viruses are variably shaped or pleomorphic. The poxviruses are the largest viruses (260 × 450 nm), and the smallest human virus is the poliovirus, which is 25 nm in diameter.

Taxonomy

Originally, viruses were classified by the diseases they caused and their host range. Now, viruses are classified in orders, families, genera, and species based on genome type (RNA or DNA), number of strands in the genome (ds or ss), morphology, and presence or absence of an envelope. Nucleotide sequence is also a valuable tool for the taxonomic placement of viruses. A summary of the clinically significant viruses is shown in [Table 29.1](#).

Viral replication

Viruses are **obligate intracellular parasites**, that is, they must be inside a living cell and use the host cell machinery to replicate. Virions absorbing or attaching to the cell surface is the first step in infecting a cell. The absorption is specific for certain cell receptors via viral *adhesion molecules*. Most host

Table 29.1 List of viruses causing human disease, based on nucleic acid characteristics and taxonomy

Genome strand	Family (subfamily)	Genus	Species
dsDNA	Adenoviridae	<i>Mastadenovirus</i>	<i>Human mastadenoviruses</i> A to G
	Herpesviridae (Alphaherpesvirinae)	<i>Simplexvirus</i>	<i>Human alphaherpesviruses</i> 1 and 2, macacine herpesvirus 1 (B virus)
		<i>Varicellovirus</i>	<i>Human alphaherpesvirus</i> 3 (varicella-zoster virus)
	(Betaherpesvirinae)	<i>Cytomegalovirus</i>	<i>Human betaherpesvirus</i> 5 (cytomegalovirus)
		<i>Roseolovirus</i>	<i>Human betaherpesvirus</i> 6
			<i>Human betaherpesvirus</i> 7
	(Gammaherpesvirinae)	<i>Lymphocryptovirus</i>	<i>Human gammaherpesvirus</i> 4 (Epstein-Barr virus)
		<i>Rhadinovirus</i>	<i>Human gammaherpesvirus</i> 8 (Kaposi sarcoma-associated virus)
	Papillomaviridae	<i>Alphapapillomavirus</i>	<i>Alphapapillomavirus</i> 1 (human papillomavirus 32)
		<i>Betapapillomavirus</i>	<i>Betapapillomavirus</i> 1 (human papillomavirus 5)
		<i>Gammapapillomavirus</i>	<i>Gammapapillomavirus</i> 1 (human papillomavirus 4)
		<i>Mupapapillomavirus</i>	<i>Mupapapillomavirus</i> 1 (human papillomavirus 1)
		<i>Nupapapillomavirus</i>	<i>Nupapapillomavirus</i> 1 (human papillomavirus 41)
	Polyomaviridae	<i>Alphapolyomavirus</i>	<i>Human polyomavirus</i> 5, 8, 9, 12, and 13
		<i>Betapolyomavirus</i>	<i>Human polyomavirus</i> 1 (BK polyomavirus), <i>Human polyomavirus</i> 2 (JC polyomavirus)
		<i>Deltapolyomavirus</i>	<i>Human polyomavirus</i> 6, 7, 10, and 11
	Poxviridae (Chordopoxvirinae)	<i>Molluscipoxvirus</i>	<i>Molluscum contagiosum virus</i>
		<i>Orthopoxvirus</i>	<i>Cowpox virus</i> , <i>Monkeypox virus</i> , <i>Vaccinia virus</i> , <i>Variola virus</i>
		<i>Parapoxvirus</i>	<i>Orf virus</i>
		<i>Yatapoxvirus</i>	<i>Yaba monkey tumor virus</i>
dsDNA, ssDNA	Hepadnaviridae	<i>Orthohepadnavirus</i>	<i>Hepatitis B virus</i>
ssDNA	Parvoviridae (Parvovirinae)	<i>Bocaparvovirus</i>	<i>Primate bocaparvovirus</i> 1 and 2 (human bocaviruses)
		<i>Dependoparvovirus</i>	<i>Adeno-associated dependoparvovirus</i> A and B
		<i>Erythroparvovirus</i>	<i>Primate erythroparvovirus</i> 1 (human parvovirus B19)
dsRNA	Picobirnaviridae	<i>Picobirnavirus</i>	<i>Human picobirnavirus</i>
	Reoviridae (Sedoreovirinae)	<i>Rotavirus</i>	<i>Rotavirus</i> A, B, C, and H
		<i>Orbivirus</i>	<i>Changuinola virus</i> , <i>Great Island virus</i> , <i>Lebombo virus</i> , <i>Orungo virus</i>
		<i>Seadornavirus</i>	<i>Banna virus</i>
	Reoviridae (Spinareovirinae)	<i>Coltivirus</i>	<i>Colorado tick fever virus</i>
		<i>Orthoreovirus</i>	<i>Mammalian orthoreovirus</i>
ssRNA	Arenaviridae	<i>Mamma renavirus</i>	<i>Lymphocytic choriomeningitis mammarenavirus</i> , <i>Lassa mammarenavirus</i> , <i>Junin mammarenavirus</i> , <i>Lujo mammarenavirus</i> , <i>Machupo mammarenavirus</i>
	Astroviridae	<i>Mamastrovirus</i>	<i>Mamastrovirus</i> (human astroviruses 1, 6, 8, and 9)
	Peribunyaviridae	<i>Orthobunyavirus</i>	<i>California encephalitis orthobunyavirus</i> , <i>Bunyamwera orthobunyavirus</i> , <i>Madrid orthobunyavirus</i>
	<i>Hantaviridae</i>	<i>Orthohantavirus</i>	<i>Hantaan orthohantavirus</i> , <i>Sin Nombre orthohantavirus</i> , <i>Puumala orthohantavirus</i>
	<i>Nairoviridae</i>	<i>Orthonairovirus</i>	<i>Crimean-Congo hemorrhagic fever orthonairovirus</i>
	<i>Phenuivirus</i>	<i>Phlebovirus</i>	<i>Rift Valley fever phlebovirus</i> , <i>Punta Toro phlebovirus</i>
	Caliciviridae	<i>Norovirus</i>	<i>Norwalk virus</i>
		<i>Sapovirus</i>	<i>Sapporo virus</i>

Continued

Table 29.1 List of viruses causing human disease, based on nucleic acid characteristics and taxonomy—cont'd

Genome strand	Family (subfamily)	Genus	Species
	Coronaviridae (Coronavirinae)	<i>Alphacoronavirus</i>	<i>Human coronavirus 229E, human coronavirus NL63</i>
		<i>Betacoronavirus</i>	<i>Betacoronavirus 1, human coronavirus HKU1, human coronavirus OC43, MERS-related coronavirus, SARS-related coronavirus</i>
	(Torovirinae)	<i>Torovirus</i>	<i>Human torovirus</i>
	Filoviridae	<i>Marburgvirus</i>	Marburg marburgvirus, Ravn virus
		<i>Ebolavirus</i>	Zaire ebolavirus, Tai Forest ebolavirus, Reston ebolavirus, Sudan ebolavirus, Bundibugyo ebolavirus
	Flaviviridae	<i>Flavivirus</i>	<i>Yellow fever virus, West Nile virus, Dengue virus, Zika virus, Japanese encephalitis virus, Langat virus, Murray Valley encephalitis virus, Omsk hemorrhagic fever virus, Powassan virus, St. Louis encephalitis virus, Tick-borne encephalitis virus</i>
		<i>Hepacivirus</i>	<i>Hepacivirus C (hepatitis C virus)</i>
	Hepeviridae	<i>Orthohepevirus</i>	<i>Orthohepevirus A (hepatitis E virus)</i>
	Orthomyxoviridae	<i>Influenzavirus A</i>	<i>Influenza A virus</i>
		<i>Influenzavirus B</i>	<i>Influenza B virus</i>
		<i>Influenzavirus C</i>	<i>Influenza C virus</i>
		<i>Influenzavirus D</i>	<i>Influenza D virus</i>
	Paramyxoviridae	<i>Respirovirus</i>	<i>Human respirovirus 1 and 3 (human parainfluenza viruses 1 and 3)</i>
		<i>Morbillivirus</i>	Measles virus
		<i>Rubulavirus</i>	<i>Human rubulavirus 2 and 4 (human parainfluenza viruses 2 and 4), Mumps rubulavirus</i>
		<i>Henipavirus</i>	Hendra henipavirus, Nipah henipavirus
	Pneumoviridae	<i>Orthopneumovirus</i>	<i>Human orthopneumovirus (human respiratory syncytial virus)</i>
		<i>Metapneumovirus</i>	<i>Human metapneumovirus</i>
	Picornaviridae	<i>Enterovirus</i>	<i>Enterovirus A (includes some coxsackieviruses)</i> <i>Enterovirus B (includes echoviruses and some coxsackieviruses)</i> <i>Enterovirus C (includes polioviruses and some coxsackieviruses)</i> <i>Enterovirus D Rhinovirus A, B, and C</i>
		<i>Parechovirus</i>	<i>Parechovirus A (human parechovirus)</i>
		<i>Hepatovirus</i>	<i>Hepatovirus A (hepatitis A virus)</i>
		<i>Lyssavirus</i>	<i>Rabies lyssavirus</i>
	Rhabdoviridae		
	Retroviridae (Orthoretrovirinae)	<i>Lentivirus</i>	<i>Human immunodeficiency virus 1 and 2</i>
		<i>Deltaretrovirus</i>	<i>Primate T-cell lymphotropic virus 1 and 2</i>
	Togaviridae	<i>Alphavirus</i>	<i>Chikungunya virus, eastern equine encephalitis virus, Venezuelan equine encephalitis virus, western equine encephalitis virus</i>
		<i>Rubivirus</i>	<i>Rubella virus</i>

CoV, Coronavirus; dsDNA, double-stranded deoxyribonucleic acid; MERS, Middle East respiratory syndrome; SARS, severe acute respiratory syndrome; ssDNA, single-stranded deoxyribonucleic acid; ssRNA, single-stranded ribonucleic acid.

cell receptors are glycoproteins, some of which include the immunoglobulin superfamily molecules (for poliovirus), acetylcholine (for rabies virus), sialic acid (for influenza virus), CD4 (for HIV), complement receptor C3d (for Epstein-Barr virus [EBV]), and angiotensin-converting enzyme 2 (for SARS CoV-2). The adhesion molecule receptor interaction determines the viral tissue tropism in the host.

The next step in viral replication is penetration. Viruses can penetrate the cell by several mechanisms. Naked virions can penetrate the cell membrane directly. Enveloped viruses can enter the cell by fusion with the plasma membrane, or by endocytosis, whereby the enveloped virus enters the cell in a cytoplasmic vacuole. Once inside the cell, the virus loses

its protein coat, releasing the genome. This process is called *uncoating*. RNA viruses usually release the genome into the cytoplasm, whereas most DNA viruses release their genome into the host nucleus. The viral genome then directs the host cell to make viral proteins and replicate the viral genome. Depending on the virus, the metabolism of the host cell may be completely stopped (as with polioviruses) or may continue at a reduced scale, as seen with influenza viruses.

The next step is the assembly or maturation of the virus particles. The capsid protein subunits aggregate to form *capsomers*, and the capsomers combine to form the capsid. The capsid and genome associate to form the nucleocapsid. The new virions are then released by lysis if they are

naked viruses or by budding if they are enveloped viruses. During budding, part of the host cell plasma membrane surrounds the viral capsid and becomes the viral envelope.

Laboratory diagnosis of viral infections

Laboratories provide different levels of service, depending on the mission, financial resources, and patient populations. All of these must be balanced to provide the most cost-effective and complete diagnostic methods that meet the needs of the clinical staff. Full-service virology laboratories provide viral culture and identification by using mammalian **cell cultures** to support the growth of viruses in clinical specimens. Although not all clinical diagnostic facilities provide full virology services, laboratories can still provide information about viral infections by utilizing rapid tests that detect specific viruses in clinical specimens. Tests can involve the detection of viral antigens by using methods such as immunofluorescence (IF) or enzyme immunoassay (EIA) and detection of viral RNA or DNA using polymerase chain reaction (PCR) assays.

Low-complexity tests with waivers from the Clinical Laboratory Improvement Act (CLIA) bring viral identification services into physicians' offices and clinics. Some laboratories limit their virology services to *viral serology*—determining the patient's immune response to viruses—rather than detecting viruses directly. Although this is sometimes useful, it usually takes several weeks after infection before these antibodies are produced, which may mean that treatment would be too late or not needed before the diagnosis is made. Molecular methods based on nucleic acid detection and amplification are being used by more clinical laboratories. This technology can detect viral infections very early in infection, and many tests are completed in less than 1 hour. Over the past 10 years, this technology has improved such that larger, more complete syndromic panels are available that can quickly identify infectious agents from many types of specimens to provide a quick, accurate diagnosis of many viral and bacterial infections at once (e.g., multiplex PCR). Since viruses tend to undergo frequent mutations, the molecular test also helps to identify different variants of the same virus to identify the virulent and more dominate strains.

Specimen selection, collection, and transport

Several different clinical specimens are suitable for the diagnosis of viral diseases. The clinical signs and symptoms of diseases often point to the target organ(s) involved, which can help determine the most appropriate specimen(s) to collect. This, combined with a basic understanding of viral pathogenesis, can help in specimen selection for each specific virus. It is important to ensure, however, that the specimen collected can be used to isolate a wide range of viral pathogens because different viruses can have overlapping clinical symptoms.

Since viral shedding is usually greatest during the early stages of infection, the best specimens are those collected as early as possible, which, in many infections, is even before symptoms occur. The sensitivity of viral culture can decrease rapidly 3 days after the acute onset of symptoms, so care must be taken to collect specimens appropriately to maximize detection and identification. Specimens should be collected aseptically.

Depending on the anatomic site and the method of collection, specimens can be sterile or nonsterile (i.e., contaminated with bacteria and/or fungi). This will determine how much specimen processing is required before viral culture. Non-culture-based test methods are typically not affected by contamination, but this varies with the system. In a normally sterile specimen, such as blood, CSF, or tissue, identification of a virus without bacterial isolates usually means that the virus is the cause of the disease.

Nonsterile specimens are obtained from sites that contain normal biota, such as the respiratory tract, genital tract, skin, or stool. These specimens may require processing to reduce contaminants and promote viral growth. Aspirated secretions are often preferable, but swabs are easier to use for collection. Swabs must be made of Dacron or rayon. Calcium alginate swabs inhibit the replication of some viruses and can interfere with nucleic acid amplification tests (NAATs). Tissue samples must be kept moist and must not be placed in transport media unless it is specifically designed for viral preservation. Often it is preferred for tissue specimens to be sent to the laboratory in a sterile container.

Viral transport medium, saline, or trypticase soy broth can be added to sterile containers to keep tissues from drying. Several viral transport media are commercially available, and most consist of a buffered isotonic solution with a protein, such as albumin, gelatin, or serum, to protect more fragile viruses. Often, antibacterial and antifungal agents are added to viral transport media to inhibit contamination by microorganisms. Samples that can be collected with viral transport media are respiratory, swab, and tissue samples. Samples that should be collected without viral transport media include blood, bone marrow, CSF, amniotic fluid, urine, pericardial fluid, and pleural fluid. The transport container should be unbreakable and able to withstand freezing and thawing.

It is optimal to process viral specimens for culture immediately. Some viruses, such as respiratory syncytial virus (RSV), are much more difficult to recover, even a few hours after collection. If specimens cannot be processed immediately after collection, they should be stored at 4 C. Specimens should not be frozen unless a significant delay (>4 days) in processing is anticipated. In this case, specimens should be frozen and held at -70 C. Specimens should never be stored at -20 C because this temperature facilitates the formation of ice crystals that will disrupt the host cells and result in loss of viral viability. Repeated freeze-thaw cycles must be avoided because they can also result in loss of viral viability. Freeze-thaw cycles are less of a problem for molecular testing since nucleic acids are able to withstand temperature changes. As a rule, repeat freeze-thaw cycles should always be avoided and the laboratory scientists must follow the guidelines provided by the manufacturer of the specific test.

Appropriate specimens for maximum recovery

For optimal recovery, specimens for viral isolation should be collected from the affected site. For example, secretions from the respiratory mucosa are most appropriate for viral diagnosis of respiratory infections. Aspirates, or surface swabs, are usually appropriate for lesions. If the intestinal mucosa is involved, a stool specimen is appropriate. However, if systemic, congenital, or generalized disease is involved,

Table 29.2 Tests available for common viral pathogens and specimens for culture

Body system affected	Antigen detection	Virus isolation	Serology	Culture specimens	Molecular testing
Respiratory tract	Adenovirus, coronavirus-2, herpes simplex virus (HSV), cytomegalovirus (CMV), influenza virus types A and B, parainfluenza virus, respiratory syncytial virus (RSV)	Adenovirus, coxsackievirus group A, coxsackievirus group B, echovirus, HSV, CMV, influenza virus types A and B, parainfluenza virus, RSV, reovirus, rhinovirus	Adenovirus, coxsackievirus group A, coxsackievirus group B, echovirus, HSV, CMV, influenza virus types A and B, parainfluenza virus, RSV	Nasal aspirate, nasopharynx (NP) or throat swabs, bronchoalveolar lavage, lung biopsy	Single marker molecular testing available or panels for typical respiratory pathogens available
Gastrointestinal tract	Adenoviruses 40 and 41, rotavirus	Adenoviruses 40 and 41, coxsackievirus group A, reovirus	Adenoviruses 40 and 41, coxsackievirus group A	Stool, rectal swab	Panels for multiple markers available
Liver			Hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis D virus (HDV), hepatitis E virus (HEV), Epstein-Barr virus (EBV)		
Cutaneous	HSV, adenovirus, varicella-zoster virus (VZV)	HSV, adenovirus, coxsackievirus group A, coxsackievirus group B, echovirus, enterovirus, measles virus, VZV, reovirus, rubella virus, vaccinia virus	HSV, adenovirus, coxsackievirus group B, dengue virus, echovirus, human herpesvirus 6 (HHV-6), measles virus, VZV, parvovirus B19, rubella virus, vaccinia virus	Vesicle aspirate, NP aspirate and stool, lesion swab	Molecular testing is available for HSV-1 and HSV-2
Central nervous system	HSV-1 and HSV-2, mumps virus, CMV, enterovirus, HHV-6, human parechovirus, VZV	Coxsackievirus group A, coxsackievirus group B, echovirus, enterovirus, poliovirus, HSV-1 and HSV-2, mumps virus	Coxsackievirus group A, coxsackievirus group B, echovirus, poliovirus, HSV, HHV-6, mumps virus	Cerebrospinal fluid (CSF), brain biopsy, NP swabs, stool	Molecular panel available for CSF that includes CMV, enterovirus, HSV-1 and HSV-2, HHV-6, human parechovirus, and VZV
Ocular	Adenovirus, HSV	Adenovirus, HSV, coxsackievirus group A, enterovirus	HSV, coxsackie group A	Corneal swabs, conjunctival scrapings	
Genital	HSV	HSV	HSV	Vesicle aspirate, vesicle swab	Molecular testing available for HSV-1 and HSV-2

specimens from multiple sites, including blood (buffy coat), CSF, and the portals of entry (oral or respiratory tract) or exit (urine or stool) are appropriate. For example, enteroviruses that cause respiratory infections could also be recovered from the stool after the respiratory shedding has ceased. In addition, enteroviruses are a major cause of aseptic meningitis and can also be isolated from urine specimens. Table 29.2 lists recommended specimens to be collected for viral diagnosis according to the body site affected. Incorrect or poor specimen collection can result in a false-negative diagnostic result.

Methods in diagnostic virology

The clinical laboratory uses three major methods to diagnose viral infections:

- Direct detection in clinical specimen
 - Microscopy to detect cytopathic effect

- Detection of virus antigens
- Nucleic acid detection
- Isolation of viruses in cell cultures
- Serologic assays to detect antibodies to virus

Each laboratory must decide on the methods to offer based on the spectrum of infections encountered, population of patients served, financial resources, and staffing. In most laboratories, a combination of several methods is used to optimize detection and reduce cost.

Direct detection

In general, direct detection methods are not as sensitive as culture methods but can offer quick results to allow rapid therapy. Many of these tests can be performed in a few minutes. Viral detection allows clinicians to make relevant decisions about therapy, infection control measures, and

hospitalization. In many cases, virology results may be available before routine bacteriology culture results are.

Microscopy

Because of their extremely small size, bright-field light microscopy is not useful for visualizing viruses. The poxviruses, however, are the largest of the human viruses (about the size of chlamydiae) and can be seen. Electron microscopy has a greater magnification and can be used to visualize virions, and it is useful to detect nonculturable viruses, such as Norwalk virus, in stool filtrates. However, electron microscopy is expensive, labor-intensive, and not a very sensitive method of detecting viruses. Therefore electron microscopy is rarely used in clinical laboratories and is more suited for large teaching or research institutions.

Many viruses produce distinctive visual changes in infected cells referred to as a **cytopathic effect (CPE)**. Although virus particles cannot be visualized, the CPE can be detected in cells collected from infected sites with bright-field microscopy. The most common method used is the Tzanck smear, which is the scraping of an ulcer base to look for Tzanck cells. Tzanck cells are large round keratinocytes that are degenerated as a result of viral infection. A Tzanck smear can detect Cowdry type A bodies from herpes simplex virus (HSV) and varicella-zoster virus (VZV) lesions. Papanicolaou (Pap) smears can reveal human papillomavirus (HPV)-associated koilocytes, which are squamous cells with an enlarged nucleus surrounded by a nonstaining halo. Rabies is sometimes diagnosed by detecting Negri bodies, which are eosinophilic cytoplasmic inclusions in neurons. However, this is an insensitive method for diagnosing rabies.

Antigen detection

IF can be a valuable tool to detect viral antigens in clinical specimens. Fluorophore-labeled antibodies against viral antigens allow direct visualization of viruses and infected cells, and some tests can amplify signals, which enhance sensitivity. In the direct fluorescent antibody (DFA) tests, cells from a patient are fixed to a microscope slide, and fluorescence-labeled antibodies are added. If viral antigens are present in the sample, the labeled antibody will bind, and fluorescence will be seen using fluorescent microscopy (see [Chapter 10](#) for a more detailed description). This test is reasonably easy to

perform and is highly specific, and can be completed in 2 to 6 hours. DFA assays are available for numerous viruses, including adenovirus, influenza A and B viruses, measles virus, parainfluenza viruses (PIVs) 1 to 4, and respiratory syncytial virus (RSV) from respiratory specimens; herpes simplex virus (HSV)-1, HSV-2, and varicella zoster virus (VZV) from cutaneous lesion material; and cytomegalovirus (CMV) from blood. The key caveat is cross-reactivity of closely related viruses to the antibodies used. Therefore it can be difficult to precisely identify the viral strain using DFA.

Many EIA tests for viral antigen detection are commercially available, and most use multi-well microtiter plate formats, which offer variable testing capacity to meet the needs of small or large laboratories. These tests can detect RSV and influenza A virus from respiratory specimens, hepatitis B virus (HBV) and HIV-1 from serum or plasma, enteric adenoviruses from feces, and HSV from cutaneous lesions and conjunctival swabs. Other tests are packaged in single-test platforms, with positive specimens detected by colorimetric or optical density changes on membrane or silicon surfaces ([Fig. 29.1](#)). Often the biggest problem with this testing is in the interpretation of a color change or the detection of a line. Some vendors now supply optical readers for these types of assays to help standardize interpretation of the results.

EIA is often less sensitive than cell cultures or IF, so negative results are confirmed with cell culture, IF, or nucleic acid-based tests. These assays are the most popular viral testing methods in hospital-based laboratories, but most EIA testing will likely be replaced by nucleic acid-based detection as it continues to become cheaper, is easier to perform, and offers the ability to detect multiple viruses simultaneously (multiplex testing).

Nucleic acid-based detection

Nucleic acid-based detection assays have shifted the focus of clinical virology. Not only can the presence or absence of a virus be determined with nucleic acid-based analysis but, depending on the assay used, a quantitative result can also be obtained. Advantages of nucleic acid-based detection assays include a much faster turnaround time (TAT); better sensitivity compared with cell culture and DFA; assays that can be quantitative; detection of nonculturable viruses such as norovirus (NoV) and hepatitis viruses; multiplex testing; and



Fig. 29.1 A, Card format rapid immunochromatographic membrane assay, BinaxNOW (Abbott Laboratories, Chicago, IL), for three common respiratory viruses: influenza A and B and respiratory syncytial virus. B, Examples of positive and negative results.

potentially genetic characterization of the virus (genotype). Overall, molecular assays lead to faster treatment and more favorable patient outcomes. Disadvantages include detection of both active and inactivated virus, higher cost, need for specialized training and more complex facilities, and lack of assays approved by the FDA. Smaller clinical laboratories often rely on sending these tests to reference laboratories at a higher cost and longer TAT.

Examples of nucleic acid–based assays include the polymerase chain reaction (PCR) and reverse transcription-PCR (RT-PCR) assays; branched DNA assay; nucleic acid sequence–based amplification (NASBA); and a combination of PCR and flow cytometry, such as the Luminex system (Luminex Molecular Diagnostics, Austin, TX) for multiplex detection. Nucleic acid hybridization assays can detect viruses from various clinical specimens. Assays are available to detect many viruses, including HPV from endocervical specimens, and classify them into types that have a high risk or a low risk for cancer. Other hybridization tests can detect CMV from blood and HBV from plasma and serum.

Numerous gene amplification techniques are available for detection of viral genomes, primarily bloodborne pathogens, such as HIV-1, HBV, hepatitis C virus (HCV), and WNV. With a dramatic increase in the incidence of West Nile fever in the United States, there has been an increased demand for WNV testing by PCR. Detection of influenza A virus by PCR assay was shown to be not only more sensitive than traditional cell culture and shell vial methods, but it also allowed earlier administration of antiviral therapy to patients, resulting in more favorable patient outcomes. A microarray assay for rapid subtyping of influenza A virus isolates is available and would be valuable in an outbreak or pandemic.

A Luminex assay to detect and type or subtype 20 different viral pathogens within 5 hours has also been described. These types of systems help epidemiologists, infectious disease physicians, and others in the public health community by rapidly identifying viral pathogens during an outbreak. Newer isothermal nucleic acid amplification technology is now becoming more prevalent (Alere, Waltham, MA); it does not require temperature cycling and can deliver results in as soon as 20 minutes with vast performance improvement over slower PCR-based assays.

In the case of global pandemics, rapid and continuous monitoring is of utmost importance. For example, a number of nucleic acid–based assays were deployed to monitor the spread of COVID-19. These include quantitative real-time PCR assays, which provide results in 2 to 3 hours, as well as isothermal assays such as the Abbott assay, which can provide a result within 5 minutes. With the emergence of mutations, it is desirable to screen for multiple variants of virus in a single assay. Several multiplex assay systems have been developed. For example, Cepheid's GenXpert, which identifies several viruses including Zika, Ebola, SARS-COV-2, and others, is widely used in clinical laboratories for a rapid and automated way of screening a large number of samples.

Isolation of viruses in cell cultures

In clinical virology, isolating viruses is still the “gold standard” against which all other methods are compared. Three methods are used for the isolation of viruses in diagnostic virology:

cell culture, animal inoculation, and embryonated eggs. The method most commonly used by clinical virology laboratories is cell culture. Animal inoculation is extremely costly and used only as a special resource in reference or research laboratories. For example, certain coxsackie A viruses require suckling mice for isolation of the virus. Embryonated eggs are rarely used; isolation of influenza viruses is enhanced in embryonated eggs, but this is accomplished more easily in cell culture.

Establishing at least a limited clinical virus isolation capability in routine laboratories can be justified, provided qualified personnel and space are available. Most of the clinical workload is for the detection of HSV in genital specimens and respiratory viruses. A significant number of common clinical viruses can often be identified within 48 hours of inoculation, including HSV, influenza A and B viruses, PIVs 1 to 4, RSV, adenovirus, and many enteroviruses.

Cell culture

The term *cell culture* is technically used to indicate culture of cells *in vitro*; the cells are not organized into a tissue. The term **tissue culture** or *organ culture* is used to denote the growth of tissues or an organ so that the architecture or function of the tissue or organ is preserved. Many clinical virologists use these terms interchangeably; however, *cell culture* is the correct term.

Cell cultures can be divided into three categories based on their longevity in cultures: primary, low passage (or finite), and continuous. **Primary cell cultures** are obtained from tissue removed from an animal. The tissue is finely minced and then treated with an enzyme, such as trypsin, to disperse individual cells further. The cells are then seeded onto a surface, such as in a flask or a test tube, to form a monolayer. With primary cell lines, only minimal cell division occurs. Cell viability is maintained by periodically removing cells from the surface, diluting them, and placing them into a new container. This process is referred to as *splitting* or *passaging*. Primary cell lines can only be passaged a few times before new cells must be obtained. An example of commonly used primary cell culture is one with primary monkey kidney (PMK) cells.

Finite cell cultures can divide, but passage is limited to about 50 generations. Finite cell lines, like primary cell lines, are **diploid**, that is, they contain two copies of each chromosome. Diploid is the normal genetic makeup for eukaryotic cells. As the number of passages increases, these cells become less sensitive to viral infection. Human neonatal lung is an example of a standard finite cell culture used in diagnostic virology.

Continuous cell cultures are capable of infinite passage and are **heteroploid**, that is, they have an abnormal and variable number of chromosomes. Sometimes the number of chromosomes is not a multiple of the normal haploid number; this is referred to as **aneuploid**. HEP2 (derived from a human laryngeal epidermoid carcinoma), A549 (derived from a human lung carcinoma), and Vero (derived from a monkey kidney) are examples of continuous cell lines used in diagnostic virology. Both HEP2 and A549 were developed from cancer tissue obtained from patients during treatment. Each laboratory must decide which cell lines to use based on the spectrum of viral sensitivity, availability, and cost. Optimally, several different cell lines will be used for a single specimen to recover different viruses that may be present, analogous

to the strategy used with media for the recovery of bacteria. Table 29.3 lists some cell culture lines commonly used in clinical virology.

Mixed or engineered cell cultures are cell lines that contain a mixture of two different cell types or are made up of cells genetically modified to make identification of viral infection easier. Mixed cell lines have been developed by combining two cell lines susceptible to certain types of viruses, such as respiratory or enteric viruses. The mixed line can have greater sensitivity to a wider range of viruses and therefore reduce the number of culture vials that must be incubated. Interpreting these mixed cell cultures is sometimes difficult, but this is well worth the effort when dealing with fastidious viruses.

Cytopathic effect on cell cultures

Some viruses produce a very characteristic CPE that can provide a presumptive identification of a virus isolated from a clinical specimen. For example, HSV grows rapidly on many different cell lines and frequently produces a CPE within 24 hours. A predominantly cell-associated virus, HSV produces a focal CPE (in which adjacent cells become infected) and plaques, or clusters of infected cells. The combination of rapid growth, plaque formation, and growth on many different cell types, such as MRC-5 (Medical Research Council cell strain 5), human fibroblasts, Vero, HEp2, mink lung, and

PMK cells, is presumptive evidence for the identification of HSV. HSV is one of the few viruses that can grow on rabbit kidney cells (Fig. 29.2); therefore it is a useful cell line for HSV detection.

CMV produces an HSV-like CPE (Fig. 29.3) but grows much more slowly and only on diploid fibroblasts. VZV grows on several types of cells, including diploid fibroblasts, A549 cells, and Vero cells. Enteroviruses characteristically produce rather small, round infected cells that spread diffusely on PMK cells, diploid fibroblasts, human embryonal rhabdomyosarcoma (RD) cells, and A549 cells. Adenoviruses also produce cell rounding (Fig. 29.4) on many cell types, including diploid fibroblasts, HEp2 cells, A549 cells, and PMK cells, but this is usually larger than that caused by enteroviruses. The rounding may be diffuse or focal, appearing like a cluster of grapes.

The respiratory viruses may not produce characteristic CPE. RSV can produce classic syncytial formation in HEp2 or MKC cells. **Syncytia** are giant multinucleated cells resulting from cell fusion in response to the RSV infection. PIV type 2, and to a lesser extent PIV type 3, can also produce syncytia. Influenza virus commonly does not exhibit a well-defined CPE. Specimens submitted for influenza virus cultures are usually inoculated onto PMK cells, LLC-MK2 (a continuous line derived from rhesus monkey kidney), or MDCK (Madin-Darby canine kidney epithelial cells) cells. Because influenza

Table 29.3 Cell cultures commonly used in the clinical virology laboratory

Virus	PMK	HDF	HEp2	RK	A549	CPE
Herpes simplex virus	–	+++	+++	+++	+++	Large, rounded cells
Cytomegalovirus	–	+++	–	–	–	Large, rounded cells
Varicella-zoster virus	–	+++	–	–	±	Foci or rounded cells; possible syncytia
Enterovirus	+	+	++	–	+	Refractile, round cells in clusters
Adenovirus	+	++	+++	–	++	Large, rounded cells in clusters
Respiratory syncytial virus	±	±	+++	–	++	Syncytia
Influenza virus, parainfluenza virus	+++	±	–	–	–	Variable—none to granular appearance

–, Negative; +, acceptable; ++, good viral recovery; +++, recommended; ±, positive or negative; A549, human lung carcinoma cell line; CPE, cytopathic effect; HDF, human diploid fibroblasts; HEp2, human laryngeal carcinoma cell line; PMK, primary monkey kidney; RK, rabbit kidney.

Modified from Costello, M. J., et al. (1993). Guidelines for specimen collection, transportation, and test selection, *Lab Med*, 24, 19.

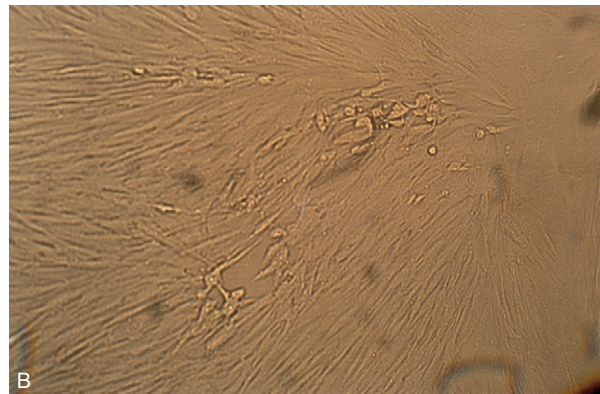
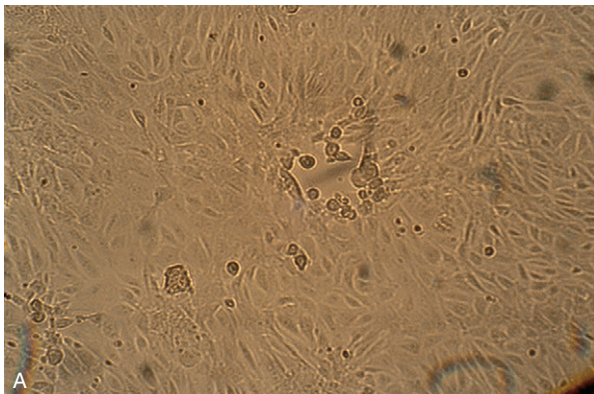


Fig. 29.2 **A**, Herpes simplex virus (HSV) from the skin, showing the cytopathic effect (CPE) in less than 1 day on rabbit kidney cells. **B**, HSV showing the CPE in less than 1 day on HeLa cells (unstained, $\times 400$).

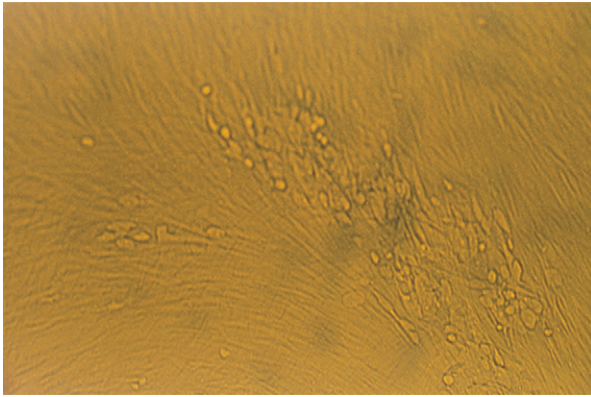


Fig. 29.3 Cytomegalovirus from cerebrospinal fluid forming cytopathic effect on diploid fibroblast cells (unstained, $\times 400$).

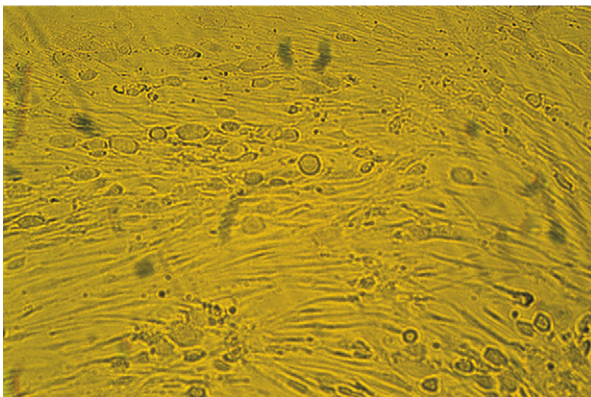


Fig. 29.4 Cytopathic effect of adenovirus on HeLa cells (unstained, $\times 400$).

viruses typically do not produce CPE, a hemagglutination or hemadsorption test is done to detect these viruses. Cells infected with influenza virus express viral **hemagglutinin (H)** protein on their surface that binds red blood cells (RBCs). In the hemadsorption test, a suspension of RBCs is added to the infected cell monolayer. If influenza virus is present, the RBCs will adsorb or stick to the infected cells. In the hemagglutination assay, supernatant from the infected monolayer containing influenza virus is mixed with a suspension of RBCs. Influenza viruses also have the H protein on their surface; therefore the RBCs will visibly agglutinate.

Fluorescent antibody stains that detect viral antigen, such as those used directly on clinical specimens, can also be used to screen cell cultures before a final negative result is reported. IF, EIA, and NAATs can also be used to detect and identify viruses in cell cultures to ensure that true positives are not missed.

Centrifugation-enhanced shell vial culture

The shell vial culture technique can more rapidly identify viruses than the traditional cell culture method. Cells are grown on a round coverslip in a shell vial. A shell vial is a small, round, flat-bottomed tube, generally with a screw cap. The shell vial, containing a cell monolayer on the coverslip, is inoculated with the clinical sample and then centrifuged to promote viral absorption. The shell vial is incubated for 24 to 48 hours, after which the coverslip is removed, and an IF assay is performed. Based on the type of clinical specimen

and suspected viruses, a variety of fluorescent-labeled antibodies can be used. A modification of this procedure is to use flat-bottomed microtiter plates. Although this is better than looking for CPE, in many cases it can be labor-intensive, and often cultures are done in duplicate, which results in reading at 24 hours then again at 48 hours, thus increasing the TAT.

Serologic assays to detect antibodies to virus

Viral serology detects circulating antibodies to viruses after exposure. This method provides limited information and has certain inherent problems (see [Chapter 10](#)). First, serologic assays measure the host response rather than directly detecting the virus. Second, the antibody-producing capabilities of human hosts differ widely from person to person. For example, despite being actively infected, immunocompromised individuals may not produce enough antibodies to be detected, and the antibodies produced may be very short-lived. This is typically seen in patients with HIV infection. Third, the antibody level does not necessarily correlate with the acuteness or activity level of the infection because this is also host dependent.

With few exceptions, paired sera (acute and convalescent) demonstrating seroconversion or a fourfold rise in titer are required to establish a diagnosis of recent infection. Therefore serologic studies are usually retrospective. Some assays can distinguish between immunoglobulin M (IgM) and immunoglobulin G (IgG); the presence of IgM indicates an acute (recent) infection. Cross-reactions with nonspecific antibodies can occur, which makes interpretation of results difficult. Interpretation is also difficult because of passive transfer of antibodies, such as in transplacental or transfusion transmission. The following are indications for serologic testing:

- Diagnosis of infections with nonculturable agents, such as hepatitis viruses
- Distinguishing between a past (IgG) or acute (IgM) viral infection
- Determination of immune status regarding rubella virus, measles virus, VZV, hepatitis A virus (HAV), and HBV
- Monitoring of patients who are immunosuppressed or have had transplantations
- Epidemiologic or prevalence studies that are critical during outbreaks to assess overall spreading and herd immunity status

Double-stranded DNA viruses

In this chapter, viruses are discussed in groups based on nucleic acid types: double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), double-stranded RNA (dsRNA), and single-stranded RNA (ssRNA) viruses. Hepatitis viruses are the only exception, and they will be discussed as one group because they do not all have the same type of nucleic acid.

Adenoviridae

Adenovirus was first isolated from adenoid tissue of children and was thus named for the initial isolation location. Human adenoviruses belong to the family Adenoviridae and

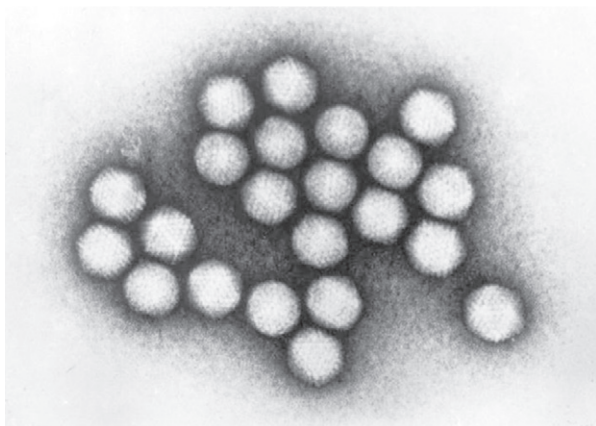


Fig. 29.5 Transmission electron micrograph of adenovirus ($\times 60,000$). (Courtesy Dr. G. William Gary, Jr., Centers for Disease Control and Prevention, Atlanta, GA.)

the genus *Mastadenovirus*. Adenoviruses are naked icosahedral viruses with dsDNA (Fig. 29.5). Adenovirus has over 60 distinct serotypes (seven subgenera, A to G), and the different serotypes are associated with numerous common clinical manifestations. The clinical manifestations seen are dependent on the age and immune status of the person infected. Adenovirus is shed in secretions from the eyes and respiratory tract. Viral shedding in feces and urine can occur for days after the symptoms have disappeared. The viruses are spread by aerosols, fomites, the oral-fecal route, and personal contact. Most infections are mild and require no specific treatment. Effective infection control measures, including adequate chlorination of swimming pools, prevent adenovirus infections, such as adenovirus-associated conjunctivitis.

The most common serotypes are 1 to 8, 11, 21, 35, 37, and 40. Although one half of all adenovirus infections are asymptomatic, the virus causes about 10% of all cases of pneumonia and 5% to 15% of all cases of gastroenteritis in children. Adenovirus infections affect the respiratory tract, eye, and gastrointestinal (GI) tract, with lesser involvement of the urinary tract, heart, central nervous system (CNS), liver, pancreas, and genital tract. The viruses can also cause epidemic keratoconjunctivitis, acute hemorrhagic cystitis, and pharyngoconjunctival fever.

Adenovirus infections occur throughout the year and affect every age group. Adenovirus serotype 14 is rarely reported but causes severe and sometimes fatal acute respiratory disease or distress (ARD) in patients of all ages. In the United States, an outbreak of adenovirus 14 was reported in four states from 2006 to 2007. The outbreak included one infant in New York and 140 additional cases from the states of Oregon, Texas, and Washington. Although no link could be found between the New York case and the other cases, all isolates were identical by hexon and fiber gene sequencing. Since 2007, adenovirus has been associated with outbreaks of ARD in U.S. military recruits and the general public. Adenovirus types 3, 4, and 7 are most associated with ARD and can be fatal. A vaccine for types 4 and 7 became available in October 2011 and is used by the U.S. military.

Adenovirus types 40 and 41 are called *enteric adenoviruses* because they cause epidemics of gastroenteritis, usually in young children, with diarrhea being a prominent feature of the

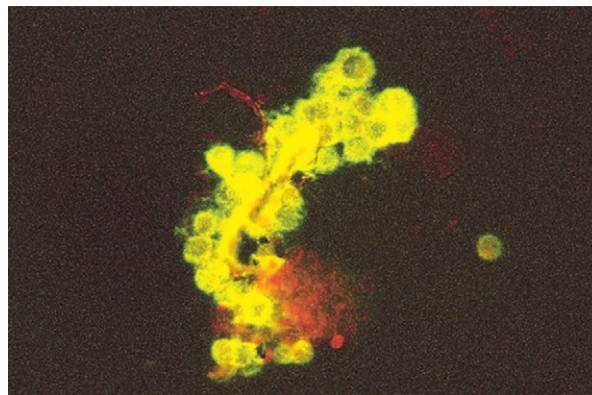


Fig. 29.6 Bronchoalveolar lavage, cytocentrifuge preparation, immunofluorescent antigen adenovirus stain, fluorescence microscopy showing prominent fluorescence of virus-infected cells ($\times 200$).

illness. There is far less vomiting and fever than with rotavirus infections. Enteric adenoviruses have a worldwide, endemic distribution, and the number of cases increases during the warmer months. These adenoviruses can be identified but not serotyped by EIA. Commercial antigen detection kits are available, and although inexpensive, they lack sensitivity. There is a molecular panel (FilmArray, BioFire Diagnostics, Salt Lake City, UT) that is specific for adenovirus types 40 and 41.

Adenoviruses are quite stable and can be isolated in human embryonic kidney and many continuous epithelial cell lines. They produce a characteristic CPE, with swollen cells in grapelike clusters. Isolates can be identified by fluorescent antibody (Fig. 29.6) and EIA methods, along with nucleic acid tests. Serotyping is accomplished by serum neutralization or hemagglutination inhibition. Electron microscopy has been used in several epidemiologic studies, but it is not routinely used as a clinical tool and is mostly only used in research settings.

Herpesviridae

The herpesviruses belong to the family Herpesviridae. The herpesviruses have a genome of linear dsDNA, an icosahedral capsid, an amorphous integument surrounding the capsid, and an outer envelope. All herpesviruses share the property of producing latency and life-long persistence in their hosts. The virus is latent between active infections. It can be activated from latency by various stimuli, including stress, physical exertion, fever, comicrobial infection, hormonal imbalance, and sunlight. Reactivation tends to be milder than the primary infection.

Eight species of human herpesvirus (HHV) are currently known:

- HSV-1, also known as HHV-1
- HSV-2, also known as HHV-2
- VZV, also known as HHV-3
- EBV, also known as HHV-4
- CMV, also known as HHV-5
- HHV-6
- HHV-7
- HHV-8, also known as Kaposi sarcoma (KS) herpesvirus

There are other herpesviruses that infect only primates, except for herpes B virus, which has produced fatal infections in animal handlers and researchers working with primates.

Herpes simplex viruses

HSV-1 and HSV-2 belong to the genus *Simplexvirus*. HSV infections are very common. By adulthood, about 80% of Americans have been infected with HSV-1. Approximately 20% of Americans have had HSV-2 infections. These figures indicate that about one in six persons in the United States has had HSV infection, and most infections are asymptomatic. Disease caused by HSV infection is generally divided into two categories: primary (first or initial infection) and recurrent (reactivation of the latent virus).

Infections are generally spread by contact with contaminated secretions. Lesions usually occur on mucous membranes after an incubation period of 2 to 11 days. Infected individuals are most infectious during the early days of a primary infection. Virus-infected cells are usually found at the edge and in the base of lesions; however, the virus can be transmitted from older lesions as well as from asymptomatic patients.

Types of infections

HSV infections occur worldwide, and infections can cause a wide spectrum of clinical manifestations. HSV-1 and HSV-2 cause the same diseases. Skin vesicles are filled with clear fluid and have a red base. The vesicles progress to pustular lesions, ulcerate, and then finally crust over.

Oral herpes

Most oral herpes infections, *herpes labialis*, are caused by HSV-1, but many cases are caused by HSV-2. The incubation period ranges from 2 days to 2 weeks. Primary infections are usually asymptomatic, but when apparent, they commonly manifest as mucosal fluid-filled vesicles inside the mouth or as ulcerations (gingivostomatitis) that can be widespread and involve the buccal mucosa, posterior pharynx, and gingival and palatal mucosae. In young adults, a primary HSV infection can involve the posterior pharynx and produce acute pharyngitis. Recurrent, or reactivation, HSV infection usually occurs on the border of the lips at the junction of the oral mucosa and skin. An early symptom of burning or pain followed by vesicles, ulcers, and crusted lesions is the typical pattern. These eruptions can result from stress or sunlight exposure and are often quite painful.

Genital herpes

Genital herpes, or *herpes genitalis*, infections are usually caused by HSV-2, although HSV-1 can cause as many as one third of the infections. Many individuals with antibodies to HSV-2 have not been diagnosed with genital herpes. The infection manifests itself in females as vesicles on the mucosa of the labia, vagina, or both. Involvement of the cervix and vulva is not uncommon. In males, the shaft, glans, and prepuce of the penis are the most affected sites. The urethra is commonly involved in both men and women. Recurrent herpes infections involve the same sites as primary infections, but the urethra is less commonly involved. The symptoms are usually less severe in recurrent disease and have a shorter duration. Herpes genitalis can as much as double the risk of sexual transmission of HIV.

Neonatal herpes

Transmission of HSV from infected mothers to neonates is less common than might be expected, but the risk of mother-to-infant transmission is 10 times higher when mothers have an unrecognized primary infection during labor and delivery. Infection can be acquired in utero (during birth) or postnatally (after birth) from hospital personnel or family members. The infection is usually transmitted during a vaginal delivery and is more severe when HSV-2 is involved compared with HSV-1. The rate of transmission is about 50% when the mother has a primary infection. Most newborns are infected by mothers who are asymptotically shedding the virus during a primary infection. The risk of transmission is very low when the mother has recurrent herpes. Cesarean delivery or suppressive antiviral therapy at delivery significantly reduces the risk of transmission. Mortality associated with disseminated neonatal disease is about 60% in treated neonates and exceeds 70% in untreated neonates.

Herpes simplex virus encephalitis

HSV encephalitis is a rare but devastating disease with a mortality rate of about 70%. In the United States, HSV encephalitis may account for up to 20% of all encephalitis cases. HSV is the leading cause of fatal sporadic encephalitis in the United States. Encephalitis is usually caused by HSV-2 in neonates and HSV-1 in older children and adults. HSV encephalitis is also associated with an immunocompromised status. Survival rates and clinical outcomes are greatly improved with IV antiviral treatment.

Ocular herpes

A herpes simplex infection of the conjunctiva can manifest itself as swelling of the eyelids associated with fluid-filled vesicles. Corneal involvement, *herpes keratitis*, can result in destructive ulceration and perforation of the cornea, leading to blindness. HSV is the most common cause of corneal infection in the United States. Fortunately, most infections involve only the superficial epithelial layer and heal completely with treatment.

Diagnosis

Diagnosis of HSV infection is best made by antigen detection or viral isolation in culture. However, smaller laboratories may perform a Tzanck smear from the lesion and look for CPE typical of HSV, and, if present, this can be confirmed using specific fluorescent antibodies to HSV-1 or HSV-2 (Figs. 29.7 and 29.8). Often, other specimen types can also be stained and observed for CPE consistent with HSV infection (Fig. 29.9). However, culture is still considered the gold standard. The best specimens for culture are aspirates from vesicles, open lesions, or host cells collected from infected sites. Culture of CSF is usually not productive. To make a culture-confirmed diagnosis of encephalitis, brain biopsy material is required.

The preferred diagnostic method for HSV in CSF is PCR. In many studies, gene amplification for HSV in CSF approaches 100% sensitivity with almost the same specificity. Some of the newer nucleic acid assays are becoming easier to perform and less costly, so it is expected that they will continue to increase in use in clinical laboratories. A diagnostic panel is available for the detection of HSV-1, HSV-2, and several other viruses and bacteria that cause meningitis (FilmArray, BioFire Diagnostics), and this test is frequently performed to reduce

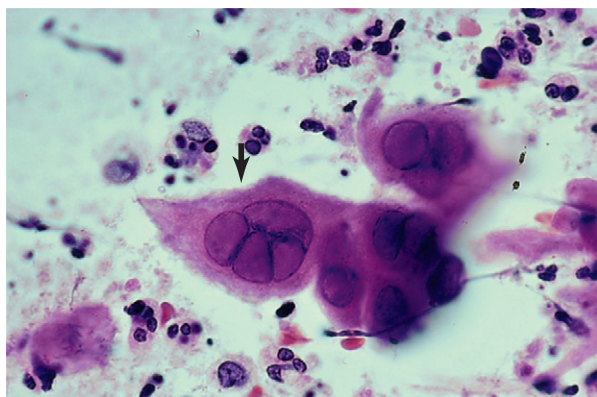


Fig. 29.7 Skin vesicle fluid, Tzanck preparation, hematoxylin and eosin stain. Multinucleated epithelial cells present (arrow). Intranuclear inclusions present, morphology consistent with herpes virus inclusions. ($\times 400$).

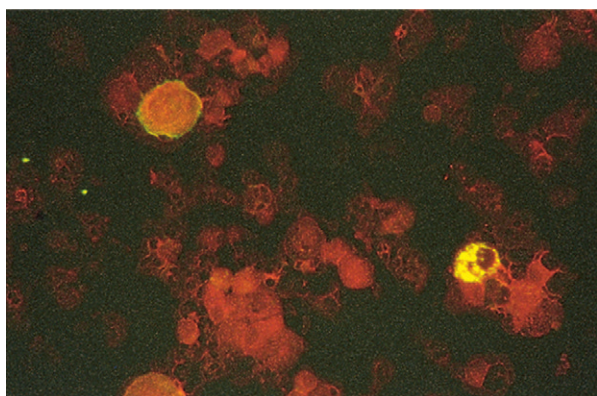


Fig. 29.8 Skin vesicle fluid, Tzanck preparation, fluorescent antibody stain for herpes simplex virus ($\times 400$).

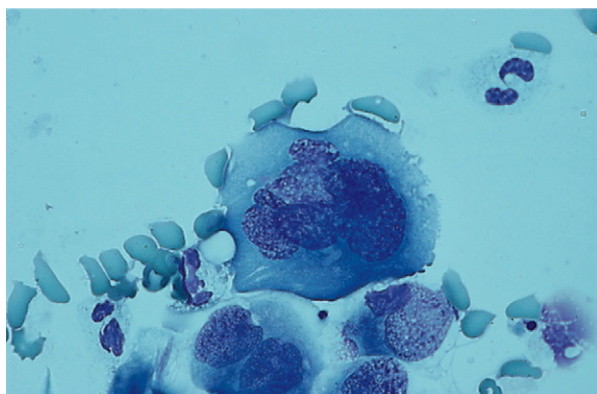


Fig. 29.9 Bronchoalveolar lavage, centrifuge preparation, rapid Wright-Giemsa stain. Multinucleated epithelial cells and intranuclear inclusions present. Morphology consistent with herpes simplex virus infection ($\times 400$).

the need for antiviral therapy, especially in infants. It is rapidly becoming the standard of care for rapid identification of viral encephalitis. There are also several HSV-1/HSV-2 standalone assays that use isothermal amplification, another form of molecular assay that does not need a thermocycler. Diagnosis can be made in a few hours, and appropriate therapy can be initiated quickly, resulting in more favorable patient outcomes.

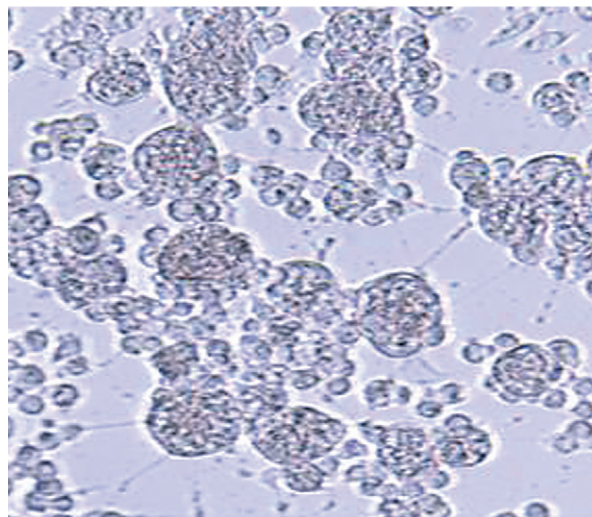


Fig. 29.10 Advanced cytopathic effect in an A549 cell line caused by herpes simplex virus infection (unstained, $\times 400$). (Courtesy Sarah Pierson.)

In culture, HSV replicates rapidly, and CPE can be seen within 24 hours (Figs. 29.2 and 29.10). Diagnosis in cell cultures is quicker than for many other viruses, but it is not as rapid as molecular assays. HSV can be isolated in numerous cell lines, including human embryonic lung, rabbit kidney, MRC-5, HEp2, and A549 cells. HSV is one of the most frequently isolated viruses in the clinical virology laboratory. Once isolated, monoclonal antibodies can be used to type the virus. Typing genital lesion isolates can be prognostic in that HSV-2 reactivation occurs more readily than HSV-1. In addition, typing genital lesions from children has been used to provide legal evidence supporting potential sexual abuse.

Commercially available engineered cell lines improve the detection of HSV. In the Enzyme Linked Virus Inducible system (ELVIS; BioWhittaker, Walkersville, MD), a gene for the enzyme β -galactosidase linked to a virus-induced promoter has been inserted into baby hamster kidney cells. If HSV-1 is present in the cell line, a viral protein will activate the promoter, resulting in β -galactosidase expression. Detection is accomplished by addition of a chromogenic reagent, which is cleaved by the enzyme produced in virus-infected cells and results in the formation of a blue color, which is easily seen by light microscopy.

Several FDA-approved, type-specific assays that differentiate antibody response to HSV are available. The tests come in a variety of formats, including EIA, strip immunoblot, and even simple membrane-based, point-of-care assays. Newer tests use recombinant or affinity-purified, type-specific glycoprotein G-1 or G-2, giving the tests the ability to distinguish between HSV-1 and HSV-2. Older-generation tests used crude antigen preparations from lysed cell culture of the virus and were shown to have cross-reactivity rates of as much as 82% in positive specimens. With this change, antigen testing has become more specific with much better results in a rapid, low cost, and easy-to-perform assay. However, because of the latent, life-long infections, many adults have antibodies to HSV-1 and HSV-2. Serologic assays can be of value in diagnosing new infections in patients without a history of infection, particularly women who are pregnant.

Cytomegalovirus

CMV is in the genus *Cytomegalovirus*, and the name originates from the enlargement of infected cells (from Latin *cyto*, meaning cell, and *mega*, meaning large). CMV is typically spread by close contact with an infected person. Most adults demonstrate antibodies against the virus, with a prevalence rate in the United States of 55% among adult women and 32% among adult men. The seroprevalence of CMV increases with age in all populations; it is highest among lower socioeconomic groups living in crowded conditions. Persons who live in overcrowded conditions can acquire CMV at an early age. The virus is shed in saliva, tears, urine, stool, and breast milk. CMV infection can also be transmitted sexually via semen and cervical and vaginal secretions and through blood and blood products. CMV infection is the most common congenital infection in the United States.

Most CMV infections are asymptomatic in the immune-competent host but can manifest themselves as a self-limiting, infectious mononucleosis-like illness, with fever and hepatitis. In immunocompromised hosts, such as transplant recipients and patients with HIV infection, CMV infection can become a significant, life-threatening, systemic disease involving almost any organ, including the lungs, liver, intestinal tract, and retina, as well as the CNS.

Congenital infections are often symptomatic and can present with serious clinical manifestations if the mother acquires the primary infection during pregnancy. Congenital infection, however, is unlikely to occur if the mother was seropositive at the time of conception. Symptomatic congenital infection is characterized by petechiae, hepatosplenomegaly, microcephaly, and chorioretinitis. Other manifestations are reduced birth weight, CNS involvement, mental impairment, deafness, and even death. CMV infection is one of the leading causes of deafness and intellectual impairment.

The diagnosis of CMV infection is best confirmed by isolation of the virus from normally sterile body fluids, such as the buffy coat of blood or other internal fluids or tissues. The virus can also be cultured from urine or respiratory secretions, but because shedding of CMV from these sites is common in normal hosts, isolation from these sources must be interpreted with extreme caution. Typically, this is done using standard stains on respiratory specimens looking for inclusions typical of CMV infection (Figs. 29.11 and 29.12).

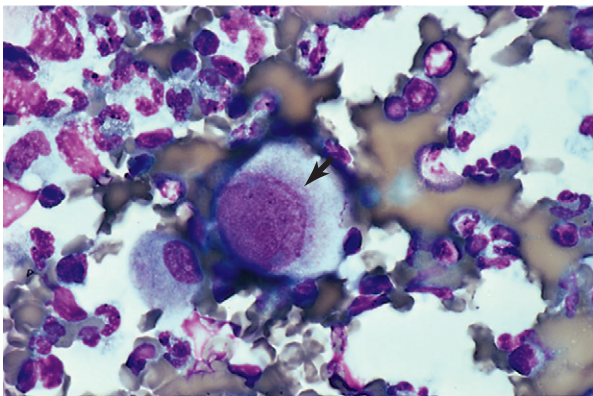


Fig. 29.11 Bronchoalveolar lavage, centrifuge preparation, rapid Wright-Giemsa stain. Enlarged pneumocyte with intranuclear inclusion. Morphology consistent with cytomegalovirus (CMV). Observe the characteristic nuclear changes for CMV. The cell and nucleus are enlarged, the nucleus is granular, and the nuclear membrane is indistinct (arrow). ($\times 400$).

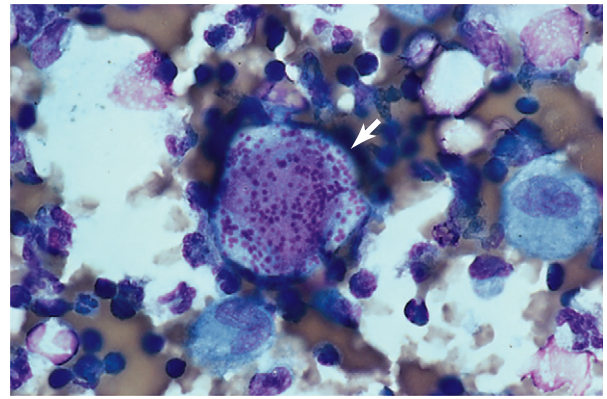


Fig. 29.12 Bronchoalveolar lavage, centrifuge preparation, rapid Wright-Giemsa stain. Enlarged pneumocyte consistent with cytomegalovirus (CMV). The large regular-sized, magenta cytoplasmic inclusions (arrow), when present, are characteristic of CMV ($\times 400$).

A viral antigenemia test is widely used by clinical virology laboratories. The antigenemia assay is specific, sensitive, rapid, and relatively easy to perform. The test is based on the immunocytochemical detection of the 65-kilodalton (kDa), lower-matrix phosphoprotein (pp65) in the nuclei of infected peripheral WBCs. The antigenemia test is more sensitive and rapid than cell culture, and it can prove helpful in assessing the efficacy of antiviral therapy. However, several newer NAATs may replace this test.

Molecular-based testing is widely used to detect virus particles in clinical samples. PCR, branched DNA, and hybridization assays are all used for diagnostic applications and blood donor screening. Meningitis panels that include CMV are available and are suitable for use in pediatric populations. A congenital infection is best confirmed by isolation of CMV from the infant within the first 2 weeks of life. Isolation after the first 2 weeks does not confirm congenital infection. Urine is the most common specimen submitted for viral detection in these patients. NAATs are the preferred method for determining viral loads.

CMV is a typical herpesvirus, but it replicates only in human cells and more slowly compared with HSV or VZV. CMV can be isolated in cell culture only by using human diploid fibroblast cell lines, such as human embryonic lung or human foreskin fibroblasts (see Fig. 29.3). The virus replicates slowly, so it may take up to 3 weeks for the CPE to appear in culture. However, the use of shell vials can reduce the time for detection to as little as 1 day. CMV produces characteristic CPE, which can sometimes be seen in clinical specimens (Fig. 29.13). Serology is not as helpful as a culture in diagnosing the infection.

Epstein-Barr virus

EBV, in the genus *Lymphocryptovirus*, causes infectious mononucleosis (Fig. 29.14). Up to 95% of adults between 35 and 40 years of age have been infected. Many children become infected with EBV and show few signs of infection. When infection with EBV occurs in adolescence, it presents as infectious mononucleosis 35% to 50% of the time. The signs and symptoms of EBV infection include sore throat, fever, lymphadenopathy, hepatomegaly, splenomegaly, and general malaise. These usually resolve within a few weeks, although

malaise can be prolonged in some cases. Complications of EBV infection include splenic hemorrhage and rupture, hepatitis, thrombocytopenia purpura with hemolytic anemia, Reye syndrome, encephalitis, and other neurologic syndromes. EBV can be recovered from the oropharynx of symptomatic

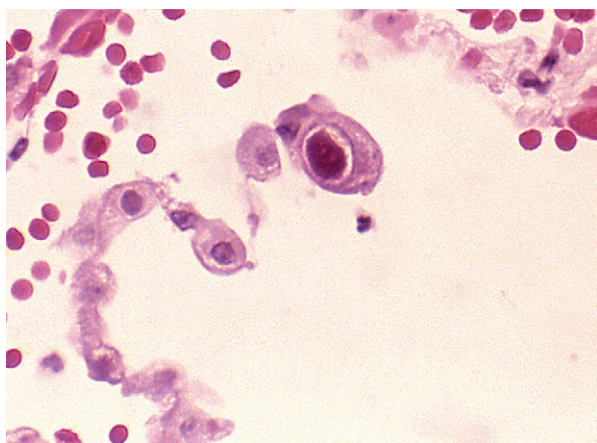


Fig. 29.13 Active cytomegalovirus lung infection in a patient with acquired immunodeficiency syndrome. Lung histopathology shows cytomegalic pneumocyte containing characteristic intranuclear inclusions (hematoxylin & eosin $\times 1000$). (Courtesy Edwin P. Ewing, Jr., Centers for Disease Control and Prevention, Atlanta, GA.)

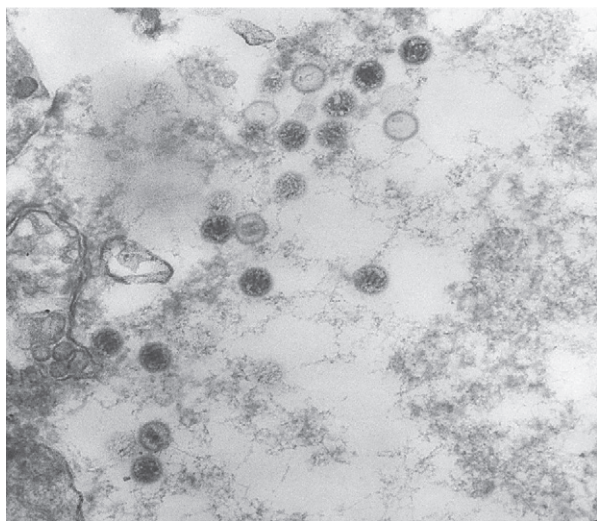


Fig. 29.14 Negatively stained transmission electron micrograph revealing the presence of numerous Epstein-Barr virions ($\times 40,000$). (Courtesy Fred Murphy, Centers for Disease Control and Prevention, Atlanta, GA.)

as well as healthy persons, who can transmit the virus to susceptible individuals via infected saliva.

The incubation period for EBV infection ranges from 2 weeks to 2 months. As with the other herpes group viruses, infection is very common and results in latency, and most adults demonstrate antibodies against the virus. Young children with the infection are almost always asymptomatic. As the age at the time of infection increases to young adulthood, a corresponding increase occurs in the ratio of symptomatic to asymptomatic infections.

Some cancers have been associated with EBV, including Burkitt lymphoma, Hodgkin disease, and nasopharyngeal carcinoma (NPC). Burkitt lymphoma is a malignant disease of the lymphoid tissue seen most commonly in African children. The virus has also been increasingly recognized as an important infectious agent in transplant recipients. The most significant clinical effect of EBV infection in these patients is the development of a B-cell lymphoproliferative disorder or lymphoma.

Viral culture for EBV requires human B lymphocytes and is beyond the capabilities of most clinical virology laboratories. Therefore laboratory diagnosis of EBV infection is often accomplished with serologic tests. EBV infects circulating B lymphocytes and stimulates them to produce multiple heterophile antibodies, including antibodies to sheep and horse RBCs. The Paul-Bunnell heterophile antibody test is an excellent rapid screening test for these antibodies, although some false-positive reactions do occur. Many rapid test kits, generally based on EIA or latex agglutination, are commercially available for detecting heterophile antibodies. These tests are 80% to 85% effective. Some false-positive test results represent patients who have had an EBV infection and still have low antibody levels. Young children can have false-negative results with the heterophile test; performing an EBV-specific antibody test on these individuals is appropriate. EBV-specific serologic tests (Table 29.4; Fig. 29.15) include the following:

- *Anti-VCA (antibodies to the viral capsid antigen)*: IgM to the VCA occurs early in the infection and disappears in about 4 weeks, so its presence indicates current infection. IgG often appears in the acute stage and will persist for life at lower titers.
- *Anti-EA IgG (IgG antibody to early antigen)*: IgG to EA can appear in the acute phase, and its presence indicates current or recent infection. The antibody usually cannot be detected after 6 months.
- *Anti-EA/D (antibody to early antigen, diffuse)*: Antibodies to EA/D appear in the acute phase, and their presence indicates current or recent infection. The antibodies

Table 29.4 Interpretation of Epstein-Barr virus serologic markers

PB	Anti-VCA IgM	Anti-VCA IgG	Anti-EA IgG	Anti-EBNA	Interpretation
—	—	—	—	—	No previous exposure to Epstein-Barr virus
+	+	+	±	—	Acute infectious mononucleosis
±	±	+	±	+	Recent infection
—	—	+	—	+	Past infection

—, Negative; +, positive; ± positive or negative; *Anti-EA IgG*, immunoglobulin G antibodies to early antigen; *anti-EBNA*, antibodies to Epstein-Barr virus nuclear antigen; *anti-VCA IgG*, immunoglobulin G antibodies against the viral capsid antigen; *anti-VCA IgM*, immunoglobulin M antibodies against the viral capsid antigen; *PB*, Paul-Bunnell antibody.

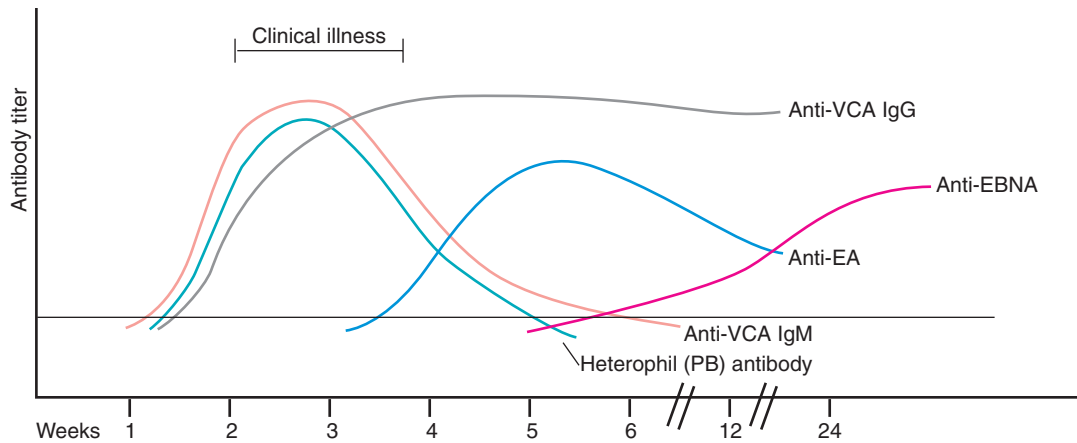


Fig. 29.15 Serologic evaluation of Epstein-Barr virus infection (infectious mononucleosis) showing the increase and decrease of detectable antibodies. *Anti-EA*, Antibody to early antigen; *anti-EBNA*, antibody to Epstein-Barr virus nuclear antigen; *anti-VCA IgG*, immunoglobulin G antibody to the viral capsid antigen; *anti-VCA IgM*, immunoglobulin M antibody to the viral capsid antigen; *PB*, Paul-Bunnell heterophile antibody.

usually cannot be detected after 6 months. Patients with NPC often have elevated levels of IgG and IgA anti-EA/D antibodies.

- *Anti-EA/R (antibody to early antigen, restricted)*: Antibodies to EA/R appear in the acute phase and disappear soon after anti-EA/D, but they can persist for up to 2 years and may be life-long in some patients. The anti-EA/R IgG antibody level is elevated in patients with Burkitt lymphoma.
- *Anti-EBNA (antibody to the EBV nuclear antigen)*: Antibodies appear about 1 month after infection, with titers peaking in 6 to 12 months.

Several molecular assays that use real-time PCR to detect and quantitate viral load are coming to market and will be key to evaluating treatment effectiveness for patients who are EBV-positive. This will especially be critical in patients who have other medical conditions that lower immune response, such as HIV infection, diabetes, or cancer.

Varicella-zoster virus

VZV is in the genus *Varicellovirus*. VZV spreads by droplet inhalation or direct contact with infectious lesions. Cell-free virus is produced at very high levels in the skin vesicles, thus the fluid from these vesicles is highly infectious. The virus causes two different clinical manifestations: varicella (chickenpox) and zoster (shingles). In the United States, more than 90% of adults have antibodies to VZV. Varicella is the primary infection and is highly contagious (Fig. 29.16). In contrast with infections caused by other herpesviruses that do not usually manifest symptoms, varicella is generally clinically apparent. It commonly appears in childhood and includes symptoms such as a mild febrile illness, rash, and vesicular lesions. Usually, the lesions appear first on the head and trunk and then spread to the limbs. The lesions dry, crust over, and heal in 1 to 2 weeks. Painful oral mucosal lesions may develop, particularly in adults.

Herpes zoster, or shingles, is the clinical manifestation caused by reactivation of VZV; it usually occurs in adults. Approximately one in three adults will develop herpes zoster in their lifetime. It is thought that the virus remains latent in the dorsal root or cranial nerve ganglia after primary

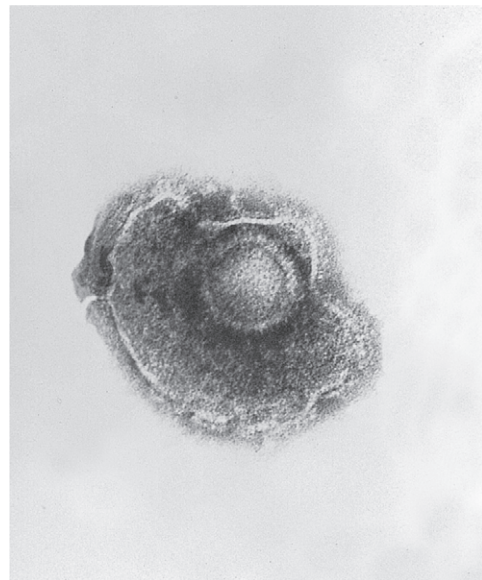


Fig. 29.16 Electron micrograph of a varicella virus ($\times 100,000$). (Courtesy Erskine Palmer and B.G. Partin, Centers for Disease Control and Prevention, Atlanta, GA.)

infection. In a small proportion of patients, the virus becomes reactivated, travels down the nerve, and causes zoster. The most common presentation is rash, followed by vesicular lesions in a unilateral dermatome pattern. These lesions may be associated with prolonged disabling pain that can remain for months, long after the vesicular lesions disappear.

VZV infection is usually diagnosed based on characteristic clinical findings. Some smaller laboratories use smears made with standard stains from lesion specimens to detect cellular inclusions typical of VZV (Fig. 29.17). In atypical cases, such as in immunocompromised patients, the diagnosis may be more difficult or questionable. In such patients, culture of fresh lesions (vesicles) or the use of fluorescent-labeled monoclonal antibodies against VZV confirms the diagnosis. VZV can be cultured on human embryonic lung or Vero cells. Cytopathic changes may not be evident for 3 to 7 days.

Recently, NAATs, such as PCR assays, have become the standard for the diagnosis of VZV disease. These assays have

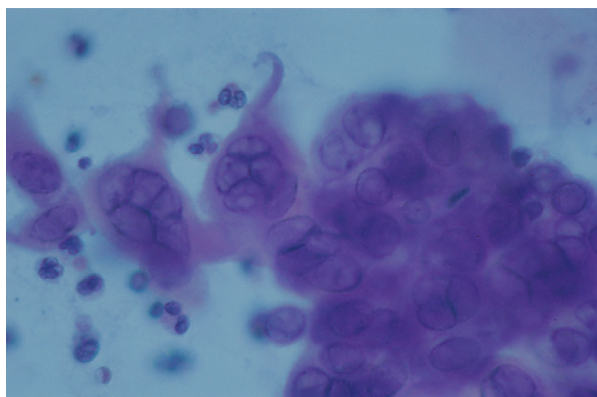


Fig. 29.17 Skin vesicle fluid, Tzanck preparation, Wright-Giemsa stain. Multinucleated epithelial cells present. Intranuclear inclusions present. Morphology consistent with herpes viral inclusions, varicella-zoster virus infection ($\times 400$).

revolutionized the diagnosis of VZV disease of the CNS and of disseminated VZV infection, especially in immunocompromised patients (e.g., HIV infection, diabetes), and the identification of herpes zoster in patients who do not develop the rash associated with VZV. The advantages of these molecular assays are that they require small specimen volumes and are highly sensitive, specific, and rapid.

An attenuated vaccine to prevent chickenpox was approved for use in the United States in 1995. Before routine use of the vaccine in children, an estimated 4 million to 5 million cases occurred annually. The vaccine is expected to give life-long immunity. In 2006, a single-dose, attenuated VZV vaccine for shingles, Zostavax (Merck, Whitehouse Station, NJ), was approved. In 2017, Shingrix (GlaxoSmithKline, Philadelphia, PA), a recombinant subunit adjuvanted vaccine, was approved; this is a two-dose vaccine. It is currently the only FDA-approved vaccine available to prevent shingles. The shingles vaccine is recommended for individuals 50 years of age and older. Antiviral treatment of VZV infection and reactivation is possible and is quite effective in reducing the infection time for patients, especially if coupled with more rapid molecular diagnostics.

Human herpesvirus 6

HHV-6 is in the genus *Roseolovirus*. The two variants of the virus, A and B, are indistinguishable serologically, but variant B appears to be the cause of disease. HHV-6 is a common pathogen. About 95% of young adults are seropositive. Studies have shown that the virus persists in the salivary glands and has been isolated from stool specimens, but most evidence indicates that saliva is the most likely route of transmission. Inhalation of respiratory droplets from and close contact with infected individuals is the primary portal of entry.

HHV-6 has been associated with the childhood disease *roseola*, which is also called *roseola infantum*, *exanthem subitum*, and *sixth disease*, reflecting its role as the sixth childhood rash. Children are protected by maternal antibodies until approximately 6 months of age. Seroconversion occurs in 90% of children between 6 months and 2 years of age. In immunocompetent individuals, most infections are mild or asymptomatic. When symptoms occur, the disease is acute and febrile; a maculopapular rash appears as the fever resolves. About 30% to 40% of infected children with symptoms experience seizures. As with all members of the family Herpesviridae, reactivation

of latent infections can become clinically significant in immunocompromised individuals. HHV-6 has also been proposed as having some involvement in the development of progressive multifocal leukoencephalopathy and multiple sclerosis.

The diagnosis of HHV-6 infection is usually made clinically. Isolation of the virus is most sensitive with lymphocyte cell culture, which is not practical for routine diagnosis. Serology may not be helpful unless paired sera are available. Patients do not usually have a positive IgM result until about 5 days after infection; IgG appears several days later. PCR and viral load testing offer the most sensitive and specific means of diagnosing primary HHV-6 infection.

Human herpesvirus 7

HHV-7 is in the genus *Roseolovirus* with HHV-6. The CD4 molecule serves as a receptor for HHV-7 to infect T lymphocytes. It also uses other receptors and has a broad range of host cells. Like HHV-6, HHV-7 is extremely common and is shed in the saliva of 75% of adults. The virus causes roseola, which is clinically identical to that caused by HHV-6. HHV-7 causes latent infections in T lymphocytes. Despite the similarities between HHV-6 and HHV-7, their antigenic diversity is such that antibodies to one virus do not protect against infection from the other. In addition, exposure to HHV-7 seems to occur later in life than exposure to HHV-6. Most 2-year-olds are seronegative for HHV-7, but most children are seropositive by 6 years of age.

HHV-7 can be isolated in culture in peripheral blood lymphocytes or in cord blood lymphocytes. Although the virus can be isolated from the saliva of healthy individuals, it is rarely isolated from peripheral blood mononuclear cells. PCR assay can detect the virus, but the ubiquitous nature of the virus can lead to difficulties in interpreting the results. Serologic results can be confusing because of cross-reactions, but patients with increasing levels of antibody to HHV-7 but not to HHV-6 may have an active HHV-7 infection.

Human herpesvirus 8

HHV-8, in the genus *Rhadinovirus*, can be detected in all forms of KS, including acquired immunodeficiency syndrome (AIDS)-related, Mediterranean, and HIV-1-negative KS, which is endemic to Africa, as well as posttransplantation KS. This association has earned it as the more common name *Kaposi sarcoma-associated herpesvirus*. It has also been shown to play a role in the development of primary effusion lymphomas and multicentric Castleman disease.

In North America and much of Europe, HHV-8 appears to be transmitted primarily through sexual contact, but studies in Africa and some Mediterranean populations suggest transmission by more casual means. The pattern of infection is like that of HSV-2, although men who have sex with men (MSM) seem to be more susceptible than heterosexuals. Prevalence ranges from close to zero in a study of Japanese blood donors to more than 50% in some parts of Africa. In HIV-positive persons, the seroprevalence can be as much as 20% to 50% higher than that of the surrounding healthy population. In the United States, as many as 20% of normal adults have antibodies to HHV-8, as do 27% of patients with HIV-1 who do not have KS and 60% of patients with HIV and KS.

Currently, the virus cannot be recovered in cell culture. PCR assay, although considered very sensitive, was shown

to be less sensitive than some immunologic assays. However, PCR assay has been used to detect the virus in various specimens, including tissue, blood, bone marrow, saliva, and semen. HHV-8 DNA or antigens are rarely detected in immunocompetent individuals, even if they are seropositive. In situ hybridization can detect HHV-8-affected tissue. The availability of commercially prepared monoclonal antibodies has made the identification of HHV-8-infected cells in various types of lesions by immunohistochemistry more common. Serologic tests are being evaluated and may soon be available.

Papillomaviridae

HPVs, the cause of papillomas (or warts), are clustered in five genera (see Table 29.1). Most clinically significant HPVs are found in the genus *Alphapapillomavirus*, which includes types infecting the genital and nongenital mucosa and genital cutaneous surfaces as well as types most often seen in human cancers. Although associated with the common wart, some HPV types are linked to cancers, including cervical cancer. There are more than 100 types of these small dsDNA viruses; more than 40 types are sexually transmitted and are known as the genital types. HPV 1, 2, 3, and 4 are thought to infect all children and young adults universally, with no significant consequences. Different HPV types exhibit different tissue tropism based on the type of epithelial cells that the viruses preferentially infect: cutaneous or mucosal. The genital HPVs are further categorized as low, intermediate, or high risk based on their association with genital tract cancers. Table 29.5 lists some HPV types and their clinical significance.

Cervical HPV lesions typically consist of flat areas of dysplasia and are often difficult to see. Rinsing the area with 5% acetic acid, which turns the lesion white, makes the lesions more visible; however, due to low sensitivity this method is not used for diagnosis. Some types of HPV will result in genital wart formation (condylomata acuminata) that can easily

be identified. Lesions can be removed by several methods, including surgery, cryotherapy, and laser.

The HPVs cannot be grown in cell cultures; therefore laboratory diagnosis of HPV infection often involves cytology sections. Cytotechnologists and cytopathologists examine Pap smears looking for koilocytes, cells with perinuclear clearing accompanied by an increased density of the surrounding rim of cytoplasm, which are indicative of HPV infection. Additional testing, such as nucleic acid probe tests, can help detect HPV DNA in endocervical cells and identify the HPV type. PCR techniques are more sensitive and have shown that HPV is present in 95% or more of invasive cervical cancers, but the presence of the virus alone is not the sole factor in cancer development. As many as one third of all college-age women are infected with HPV, and most develop only subclinical infections. Because finding HPV in cervical tissue is not the sole predictor of invasive disease, there is some debate about whether it is useful to look routinely for the virus in cervical specimens.

A quadrivalent vaccine, Gardasil (Merck, Kenilworth, NJ), against HPV types 6, 11, 16, and 18 to prevent cervical cancer was approved by the FDA in 2006 for females 9 to 26 years of age; the vaccine was approved for use in males in 2009. A second vaccine that protects against HPV types 16 and 18, Cervarix (GlaxoSmithKline), was approved for use in women in 2009. HPV types 16 and 18 are linked to most cervical cancers and other HPV-associated cancers. There has been controversy about the use of these vaccines because it is recommended that they be administered at a young age, preferably before sexual activity.

Poxviridae

Poxviruses belong to the family Poxviridae, and they are among the largest of all viruses. They are about 225 to 450 nm long and about 140 to 260 nm wide. These viruses have a characteristic brick shape and contain a dsDNA genome. Variola

Table 29.5 Human papillomaviruses and their clinical significance		
Human papillomavirus type	Clinical manifestation	Association with malignancy
Cutaneous		
1	Plantar warts	None
2–4	Common warts	None
5, 8, 9, 12, 14, 15, 17, 19–25, 36–38	Flat and macular warts	>30% of patients with epidermodysplasia verruciformis (a rare autosomal disease) with types 5, 8, 14, 17, and 20 develop malignancy
26–29, 34	Common and flat warts	Frequent, especially in immunocompromised patients
Mucosal		
6, 11	Papillomatosis, primarily laryngeal, also upper respiratory tract and condylomata acuminata (genital warts)	Low risk
42, 43, 44	Condylomata acuminata	Low risk
31, 33, 35, 51, 52	Condylomata acuminata	Intermediate risk
16, 18, 45, 56, 58, 59, 68	Condylomata acuminata	High risk
Modified from Gravitt, P. E., & Ginocchio, C. C. (2011). Human papillomaviruses. In J. Versalovic, et al. (Eds), <i>Manual of clinical microbiology</i> (10th ed., p. 1612). Washington, DC: ASM Press; 2011.		

virus belongs to the genus *Orthopoxvirus*. Members of the genus include **vaccinia virus** (the smallpox vaccine strain), monkeypox virus, cowpox virus, among others.

Variola virus

Variola virus causes smallpox, a disease that was common throughout early history (Fig. 29.18). Edward Jenner demonstrated the efficacy of vaccination against smallpox in 1796, which ultimately led to control and eradication of the disease. The last reported case of smallpox in the United States was in 1949, and the last case of smallpox worldwide was in Somalia in 1977. After decades of aggressive efforts toward vaccination, education, and eradication, the WHO officially declared the world smallpox free in May 1980. Although extinct in nature, smallpox virus is known to be maintained in at least two locations: in the United States at the Centers for Disease Control and Prevention (CDC) and in Russia. These viruses are maintained under strict security measures, and their very existence remains a point of contention between scientists who advocate their destruction and those who wish to continue studying the virus. The two countries continue to defend maintaining these stocks on the grounds that further study is needed to produce better vaccines and as countermeasures to bioterrorism.

Typically, smallpox is characterized as a synchronous progressive rash accompanied by fever. The incubation period is approximately 10 to 17 days. The patient becomes febrile, and oral lesions can appear. At this point, the patient is infectious. Within 24 to 48 hours, a faint macular rash develops on all parts of the body. However, lesions are present in greater concentration on the head and limbs (centrifugal distribution), including the palms and soles. The macular rash progresses into papules, then vesicles, and finally into pustules that resemble chickenpox lesions. Pustules are deeply embedded into tissues. All lesions change at the same time—hence the term *synchronous*. Infected persons with dark skin tones typically scar as a result of this infection because of the depth of the pustules. The mortality rate for smallpox was, on average, 30%. Other forms of smallpox, including flat and hemorrhagic smallpox, occurred rarely and were almost always fatal.

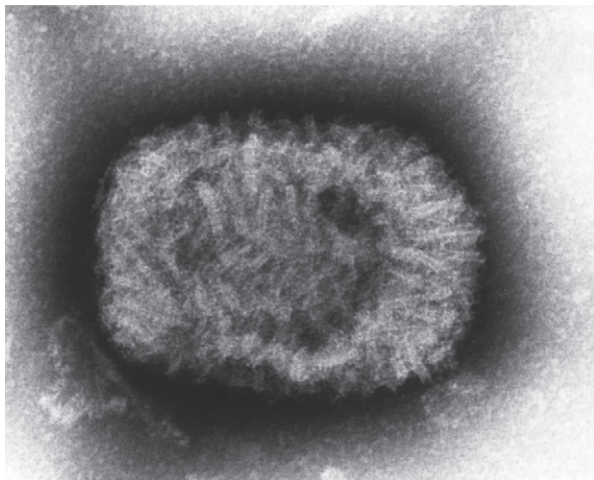


Fig. 29.18 Negatively stained transmission electron micrograph of the smallpox (variola) virus ($\times 100,000$). (Courtesy J. Nakano, Centers for Disease Control and Prevention, Atlanta, GA.)

Routine vaccination against smallpox in the United States ended in 1972, and other countries have also stopped their vaccination programs. Two vaccines are approved for use in the United States: ACAM2000, manufactured by Sanofi Pasteur Biologics (Canton, MA), and JYNNEOS, produced by Bavarian Nordic (Denmark). Although smallpox was eradicated with the use of a relatively safe vaccine, most of today's population is now susceptible to infection because vaccination is thought to offer protection for only up to 10 years. Because most humans are now susceptible to variola virus, it remains a threat as a bioterrorism weapon. Most military personnel moving into areas of conflict receive the vaccine, with little to no ill effects. If an outbreak of smallpox were to occur, there is sufficient stock of smallpox vaccine to mount an effective vaccination program in the United States. The antiviral compounds cidofovir and brincidofovir have been shown to be effective in combating disease in humans. More information on smallpox as an agent of bioterrorism can be found in [Chapter 30](#).

Monkeypox virus

Monkeypox was first described in primates in 1958, with the first case of human monkeypox in 1970. In 2003, a multistate outbreak of monkeypox occurred in the United States. It is thought that the virus was introduced into the country by rodents imported from Africa (Gambian rats). Human monkeypox infection occurs primarily in central and western Africa. Human infections are rare but result in a vesicular, pustular febrile illness that is very similar to smallpox. Monkeypox infections are less severe in humans compared with smallpox, and mortality rates are significantly lower.

In 2022, an outbreak of monkeypox occurred in 75 countries. The WHO reported 18,095 laboratory confirmed and suspected cases and 5 deaths between January 1 and July 22, 2022. The majority of the cases (72%) were reported in the European region, although the United States reported more cases than any other country. In July, the WHO declared monkeypox a global emergency. It has been suggested that monkeypox virus might have been circulating below detectable levels and maintained undetected human-to-human transmission for a period of time. It is unknown how people in this outbreak were exposed. Data suggest it is transmitted by close personal contact, and a number of cases have been found in gay, bisexual, and other men who have sex with men.

Vaccination for smallpox provides some degree of protection against monkeypox virus. There has been a concerning rise in the number of cases of monkeypox infection in non-endemic countries in recent years, indicating a weakening of protective immunity or emergence of mutant strains that are not protected by the smallpox vaccination.

Single-stranded DNA viruses

Parvoviridae

The smallest of the DNA viruses are the family Parvoviridae, which are naked ssDNA viruses that measure about 22 to 26 nm in diameter. Parvovirus B19 is the principal human pathogen in the family. It is classified in the genus *Erythrovirus*. Parvovirus B19 was named after the serum sample (number 19 of panel B)

in which the initial viral isolate was observed by electron microscopy.

Infections range from asymptomatic to potentially fatal. The most recognized syndrome is erythema infectiosum, more commonly referred to as *fifth disease*. Patients with erythema infectiosum experience a prodrome of fever, headache, malaise, and myalgia, with respiratory and GI symptoms (nausea and vomiting). The prodromal phase lasts a few days, after which a rash often appears. The rash gives a slapped cheek appearance and then spreads to the trunk and limbs. The rash occurs more commonly in children than in adults, lasts up to 2 weeks, and can recur after exposure to heat and sunlight. Adults may also experience arthralgia, arthritis, or both. In some cases, the connective tissue manifestation occurs without the prodrome or rash stage. Most infections occur in children and adolescents, and 80% of adults are seropositive by 65 years of age.

Parvovirus B19 viremia can cause transient aplastic crisis, a self-limiting erythropoietic arrest. Erythroid precursor cells contain a receptor for the virus, allowing viral infection and replication. The disease is characterized by a decrease in RBC production in bone marrow. In otherwise normal individuals, this decrease results in a short-lived anemia. The disease can be severe; complications include viremia, thrombocytopenia, granulocytopenia, pancytopenia, flulike symptoms, and congestive heart failure. Within about 1 week, reticulocytosis occurs, and the patient recovers. Persons with weakened immune systems caused by HIV infection, organ transplantation, cancer, or leukemia are at risk for serious complications from fifth disease.

Parvovirus B19 viremia creates a risk for fetuses and blood transmission in blood units. In utero infection can cause hydrops fetalis resulting from anemia. Although the most vulnerable period for the fetus is the third trimester, most women exposed to the virus do not develop an acute disease, and few infections result in loss of the fetus. The infection usually does not require therapy other than relief of symptoms, such as anti-inflammatory agents for painful joints and generalized aches.

A novel parvovirus was described in 2005. It was named the *human bocavirus* (HBoV) and causes a variety of upper and lower respiratory tract illnesses. Other viruses related to the first HBoV isolate, namely HBoV2, HBoV3, and HBoV4, have been detected principally in human feces. All of these viruses have been found in patients with gastroenteritis and in healthy controls, with HBoV2 found most often. HBoV is closely related to the bovine parvovirus and canine minute virus, both members of the genus *Bocavirus* in the family Parvoviridae. Clinical symptoms of HBoV infection include cough, rhinorrhea, fever, difficulty breathing, diarrhea, conjunctivitis, and rash. HBoV has been increasingly present as a co-infection with RSV and human metapneumovirus (HMPV). Some studies have indicated a potential link to HBoV respiratory illness and gastroenteritis. However, researchers concluded that HBoV is shed in high quantities in stool, but its link to gastroenteritis has not been demonstrated.

HBoV infection is highest during the winter months and has been detected worldwide in 5% to 10% of children 7 to 18 months of age with upper and lower respiratory tract infections. Since its first description, HBoV has been described in at least 19 countries on 5 continents. HBoV was detected less frequently than common respiratory agents (e.g., influenza virus, parainfluenza virus, adenovirus, RSV) in South African

children. Detection of HBoV has improved with the development of sensitive and specific RT-PCR assays.

Double-stranded RNA viruses

Reoviridae

Rotaviruses

Rotaviruses are naked viruses about 75 nm in diameter, with two protein layers surrounding the capsid. They belong to the genus *Rotavirus*. Rotaviruses are the most common cause of viral gastroenteritis in infants and children. Gastroenteritis is a major cause of infant death and failure to thrive. Rotaviruses have a worldwide distribution and in 2016 caused an estimated 258 million cases of diarrhea and 128,500 deaths in children less than 5 years of age. Death rates have declined due to increased availability of a vaccine. However, rotavirus accounted for 29% of all diarrhea deaths in children younger than 5 years of age. Most outbreaks occur in the winter months in the temperate zones and year-round in subtropical and tropical regions.

Rotaviruses are spread by the fecal-oral route and have an incubation period of 1 to 4 days. Symptoms generally occur suddenly and include vomiting, diarrhea, fever, and in many cases, abdominal pain and respiratory symptoms. Vomiting and diarrhea can cause rapid loss of fluids and fatal dehydration. The rotavirus replicates in the epithelial cells in the tips of the microvilli of the small intestine. The microvilli are stunted, and adsorption is reduced. The virus is shed in large quantities in the stool and can cause healthcare-associated outbreaks in the absence of good hygiene. Although the rotavirus is present in large numbers in feces, it can be isolated only with special procedures. Enzyme-linked immunosorbent assay (ELISA) and latex agglutination tests detect the viral antigens in fecal material. Rapid membrane-bound colorimetric tests are also available. Electron microscopy examination of stool samples can be used; however, as mentioned previously, this method is not very sensitive.

With the introduction in 2006 of a human-bovine rotavirus vaccine, RotaTeq (RV5; Merck), a delay in the onset of rotavirus season was seen from mid-November to late February. RotaTeq protects against five strains of RSV and is a series of three oral vaccines administered beginning at age 6 to 12 weeks. A second vaccine, Rotarix (RV1; GlaxoSmithKline), was approved in June 2008. In clinical trials, both vaccines were shown to be safe and effective. During approximately the first year of an infant's life, the rotavirus vaccine prevented 85% to 98% of severe rotavirus illness episodes and prevented 74% to 87% of all rotavirus illness episodes, which was a significant reduction in both populations.

Colorado tick fever virus

The genus *Coltivirus* contains the Colorado tick fever virus that causes a dengue-like infection in the western United States and Canada. It is an 80-nm spherical particle with two outer shells containing 12 RNA segments. Because Colorado tick fever is not reportable, actual numbers of cases are unknown. It is thought to be one of the most common diseases transmitted by ticks in the United States. Viruses transmitted by arthropods, such as ticks and mosquitoes,

are referred to as **arboviruses**. The vector for the infection is *Dermacentor andersoni*, which has many hosts in nature, including deer, squirrels, and rabbits. Infected individuals develop fever, photophobia, myalgia, arthralgia, and chills. As with dengue fever, patients can also have a biphasic fever with a rash, and children can experience hemorrhagic fever. No commercially produced laboratory tests are available, but recombinant immunoassays to detect Colorado tick fever IgG have been developed. Some nucleic acid-based assays using RT-PCR in research laboratories are becoming available and may be helpful in the future. Many primary health care providers rely on ruling out other tickborne diseases to diagnose Colorado tick fever.

Single-stranded RNA viruses

Arenaviridae

The arenaviruses get their name from the Latin *arena*, meaning “sand.” Under an electron microscope, arenaviruses appear sandy and granular. There are 43 named arenaviruses; the family includes many species that cause hemorrhagic fever. Arenaviruses are commonly divided into two complexes: Old World and New World. The New World complex includes the common human pathogens Tacaribe, Junín, and Machupo viruses, among several others. The Old World complex contains the arenaviruses commonly associated with human infection, the lymphocytic choriomeningitis (LCM) virus and the Lassa viruses, and also the benign Lassa-like Mopeia, Mobala, and Ippy viruses. Recently, a new arenavirus was identified from humans, the Lujo virus, during an outbreak of human fatal hemorrhagic fever, in which person-to-person transmission was documented. Some of the arenaviruses have not yet been isolated and are known only from molecular sequencing data.

The first arenavirus to be described was LCM virus in 1933. The first arenavirus found to cause hemorrhagic fever was Junín virus, which causes Argentine hemorrhagic fever. In 1969, Lassa virus was isolated in Africa, and it became the basis of the novel *Fever* by John Fuller. The book details the emergence of Lassa fever, which in retrospect is eerily like the emergence of other hemorrhagic fevers, including Ebola virus (EBOV) disease, which would be identified later in Africa.

The arenaviruses infect rodents, and humans are exposed to the disease by zoonotic transmission. All have been isolated from rodents of the family Muridae. The rodents are infected for long periods and typically do not become ill, but they shed virus in urine, feces, and saliva. In some parts of the United States, as many as 20% of *Mus musculus* mice carry LCM virus. Pet hamsters are also reservoirs. Humans become infected when they inhale the aerosolized virus or contact fomites. LCM virus causes flulike illness; about 25% of infected individuals develop meningitis.

Lassa virus is the most well-known of the arenaviruses. Most exposed individuals develop an asymptomatic infection, but some patients experience fever, headache, pharyngitis, myalgia, diarrhea, and vomiting. Some patients develop pleural effusions, hypotension, and hemorrhaging. CNS involvement includes seizures and encephalopathy. The mortality rate is about 15% for patients needing hospitalization. West African nations are most affected, with more

than 200,000 cases and approximately 3000 deaths occurring annually.

Spread of the virus through airline travel by people from endemic areas highlights the vulnerability of large populations; this combined with the resurgence of the infection in Nigeria has prompted vaccine development efforts. Most cases of Lassa fever are community acquired, primarily through contact with excretions from the multimammate rat *Mastomys natalensis*, which, once infected, sheds the virus throughout its life. Humans inhale the aerosolized virus or contract the virus directly through breaks in skin. Lassa virus is present in throat secretions, can be transmitted from person to person, and can also be transmitted through sexual contact and nosocomially. If therapy begins within the first 6 days of exposure, Lassa virus infection can be effectively treated with the antiviral drug ribavirin. Diagnosis of Lassa virus infection is typically made with ELISA to detect IgM and IgG antibodies.

Bunyavirales

The order Bunyavirales includes the families Peribunyaviridae (genus *Orthobunyavirus*), Nairoviridae (genus *Orthonairovirus*), and Phenuiviridae (*Phlebovirus*), all of which are classified as arboviruses. These viruses replicate initially in the gut of the arthropod vector and eventually appear in saliva. The arthropod transmits the virus when feeding on the blood of vertebrate hosts, including humans. After a few days, the infected host usually develops an asymptomatic viremia; however, some hosts become febrile, which is far less common. Most members of the order Bunyavirales cause a febrile illness, hemorrhagic fever, or encephalitis. Rift Valley fever virus targets the brain and liver to cause encephalitis and hepatitis. La Crosse virus (LACV) and California encephalitis virus cause encephalitis, and Crimean-Congo hemorrhagic fever (CCHF) virus infects the vascular endothelium and liver. The hantaviruses, which belong to the family *Hantaviridae* (genus *Orthohantavirus*) within the order Bunyavirales, do not infect arthropod hosts. They are rodent-borne viruses. Hantaviruses typically affect the peritoneal cavity, thoracic cavity, kidneys, or lungs.

The CCHF virus causes a high-mortality infection in humans. Infection begins with fever, myalgia, arthralgia, and photophobia. Patients exhibit mental status changes, ranging from confusion and agitation to depression and drowsiness. Petechiae and ecchymoses can form on mucosal surfaces and the skin. The patient may bleed from the bowel, nose, and gums. About 30% of patients die. Others begin recovering after about 10 days of illness. Healthcare-associated transmission of CCHF virus has been reported.

In the United States, LACV is a reportable disease. About 50 to 150 severe CNS diseases are reported annually. The incidence is underestimated because the disease manifests as a nonspecific fever, headache, nausea, vomiting, and lethargy. The disease is commonly found in children and usually develops in the summer, frequently referred to as the *summer flu* or *summer cold*. Because serologic tests for LACV are not offered in most laboratories and because this disease has very low mortality (about 1%), a definitive diagnosis is not often made.

The genus *Orthohantavirus* includes Hantaan virus, Seoul virus, Puumala virus, and Dobrava virus, which cause the disease *hemorrhagic fever with renal syndrome* (HFRS). These viruses are present in Asia and Europe, except for Seoul virus,

which is found worldwide. Hantaviruses endemic to Europe and Asia are called *Old World hantaviruses*. Puumala virus is the most common member of this genus in Europe and causes a mild form of HFRS, called *nephropathia epidemica*. Viruses causing HFRS target the kidneys. Patients develop a febrile prodrome and enter a phase of fever and shock, accompanied by oliguria. As the patient recovers, the kidneys gradually regain function. The mortality rate for HFRS is 1% to 15%.

In 1993, two adults from the same household in New Mexico died of an unusual respiratory illness. Serologic testing indicated that these patients had been exposed to an unknown agent that was antigenically related to one of the Asian hantaviruses despite the different clinical presentation. Serology surveys also determined that 30% of the deer mice tested in the New Mexico area were seropositive for the same unknown virus. The virus was ultimately characterized as a new hantavirus and was named the *Sin Nombre* (“without a name”) virus (SNV). The disease caused by this virus was named *hantavirus pulmonary syndrome* (HPS). Molecular techniques were subsequently developed to detect many new hantaviruses in the Americas (Table 29.6), sometimes called the *New World hantaviruses*.

SNV is transmitted through inhalation of contaminated aerosolized mouse urine, saliva, and feces. Generally, person-to-person transmission does not occur with hantavirus. Patients with HPS have a 3- to 5-day febrile prodrome, with fever, chills, and myalgia. Patients then enter a phase of hypotensive shock and pulmonary edema. The patient develops tachycardia, hypoxia, and hypotension. In severe cases, the patient can develop disseminated intravascular coagulation. The mortality rate for HPS is about 50%. Treatment for HPS is primarily supportive. No FDA-approved laboratory tests for the identification of a hantavirus infection are available; however, some European tests are being evaluated. Some

state health laboratories and the CDC perform EIAs to detect anti-SNV IgM and IgG antibodies, which may provide some useful information on the infection. Immunohistochemistry is a sensitive method used to detect hantavirus antigens in the capillary endothelium; a high concentration of antigens is found in capillary tissue specimens from the lung.

Caliciviridae

The family Caliciviridae contains 10 genera; *Norovirus* and *Sapovirus* are the most clinically significant. NoVs (Fig. 29.19) are the most common cause of infectious gastroenteritis in the United States, accounting for as many as 19 to 21 million cases annually and 900 deaths. Most deaths occur in adults 65 years of age and older. Until recently, these small, round ssRNA viruses, 27 to 30 nm in diameter, were called *Norwalk-like viruses*, *caliciviruses*, and *small round structured viruses*. They are currently placed in the genus *Norovirus*. They cause outbreaks of acute gastroenteritis in schools, colleges, nursing homes, and families as well as on cruise ships and in resort areas. NoVs have been found in drinking water, swimming areas, and contaminated food. Transmission is most commonly foodborne, although waterborne and person-to-person transmission can be significant.

The incubation period is 24 to 48 hours; the onset of severe nausea, vomiting, diarrhea, and low-grade fever is abrupt. The infection rate can be as high as 50%. The illness usually subsides within 72 hours. Immunity may be short-lived, leading to the potential for multiple infections throughout life.

The viruses cannot be grown in culture, so diagnosis relies on electron microscopy, immune electron microscopy, and real-time PCR. RT-PCR is now the most used diagnostic assay for detecting NoV. This assay detects viral RNA and can be used to test stool, vomitus, and environmental samples. Stool is the best sample to use to detect NoV. It should be collected when a person has acute illness, within 48 to 72 hours after onset of symptoms. In some cases, NoV can be detected in stool samples collected 2 weeks after recovery. Several EIAs for detecting NoV in stool samples are available. The FDA has approved an EIA for detecting NoV during outbreaks. However, at this time, EIAs are not sensitive enough for diagnosing individuals suspected of being infected.

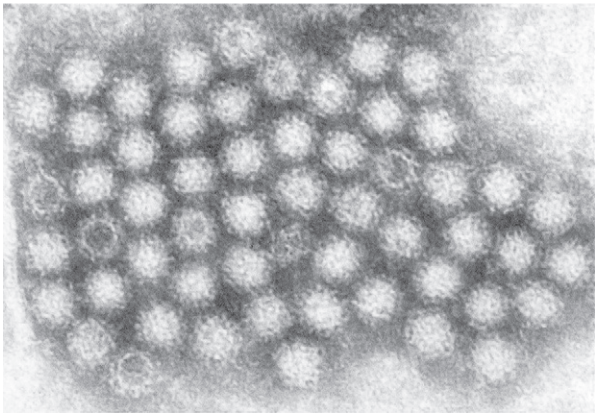


Fig. 29.19 Transmission electron micrograph revealing the ultrastructure morphology of norovirus virions ($\times 100,000$). (Courtesy Charles D. Humphrey, Centers for Disease Control and Prevention, Atlanta, GA.)

Table 29.6 Hantaviruses that cause hantavirus pulmonary syndrome		
Hantavirus	Host	Location
Sin Nombre	<i>Peromyscus maniculatus</i> (deer mouse)	United States, western Canada
Black Creek Canal	<i>Sigmodon hispidus</i> (cotton rat)	United States, South America
Bayou	<i>Oryzomys palustris</i> (marsh rice rat)	Southeastern United States
Monongahela	<i>Peromyscus maniculatus</i> (deer mouse)	Eastern United States
New York	<i>Peromyscus leucopus</i> (white-footed deer mouse)	New York
Oran	<i>Oligoryzomys longicaudatus</i> (long-tailed pygmy rice rat)	Argentina
Andes	<i>Oligoryzomys longicaudatus</i>	Argentina, Chile
Lechiguanas	<i>Oligoryzomys flavescens</i> (yellow pygmy rice rat)	Argentina
Laguna Negra	<i>Calomys laucha</i> (vesper mouse)	Paraguay, Bolivia

Sapporo viruses are small (30–35 nm in diameter) diarrheagenic viruses distinguished by a cup-shaped morphology. They usually cause diarrhea and vomiting in infants, young children, and older adults. Originally discovered in Sapporo, Japan, in 1977, these viruses are detected by electron microscopy, molecular methods (e.g., RT-PCR), and/or immunologic methods (e.g., ELISA).

Coronaviridae

Coronaviridae is one of seven families within the order Nidovirales. Of the 30 recognized species within the subfamily Coronavirinae, six are found in humans. Two human species are found in the genus *Alphacoronavirus*, and the remaining four belong to the genus *Betacoronavirus*, along with many other mammalian coronaviruses (CoVs). Many new CoVs have been discovered recently and analyzed genetically. This has resulted in hundreds of new CoV genome sequences, which has led to reorganization of the taxonomic structure and the current phylogenetic relationship of the species. CoVs have large, linear, positive-stranded RNA genomes, ranging from approximately 25 to 32 kilobases (kb) in size, and they are enveloped, helical viruses. They were first identified by electron microscopy and were named because the distinctive club-shaped projections on their surface resemble a crown (Fig. 29.20). CoVs infect several different animals; however, most individual strains of virus typically infect only a single animal species. Some can infect more than one related species.

Four strains of CoV are considered endemic to the United States. These strains are associated with upper respiratory tract infections and occasionally lower respiratory tract infections in people of all ages. One strain in particular has been linked to croup in children. CoVs may be responsible for 15% of coldlike infections in adults, but higher seroconversion rates have been seen in children. A few CoVs are responsible for a small percentage of pediatric diarrhea cases. In general, the illness lasts about 1 week, and blood may appear in the stool.

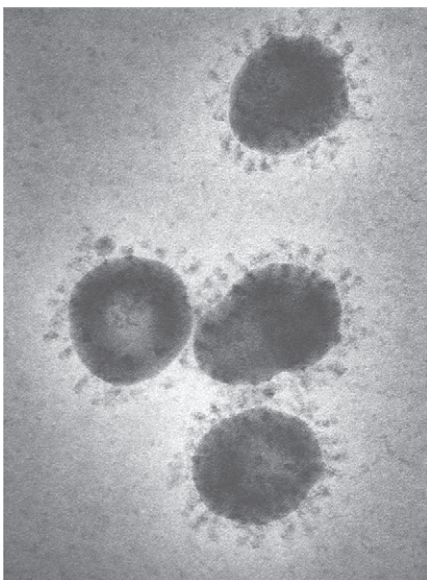


Fig. 29.20 Transmission electron micrograph of the coronavirus. This virus derives its name from the fact that under electron microscopy, the virion is surrounded by a corona, or halo ($\times 100,000$). (Courtesy Fred Murphy and Sylvia Whitfield, Centers for Disease Control and Prevention, Atlanta, GA.)

A novel CoV was the causative agent of a pandemic respiratory disease that emerged from Hong Kong in late 2002. During a 6-month period, the infection spread rapidly to 26 countries in Asia, Europe, South America, and North America. The virus infected at least 8000 people and resulted in a mortality rate of approximately 10%. The disease was characterized by high-grade fever, pneumonia, and in some patients, acute respiratory distress syndrome (ARDS). The disease was ultimately termed *severe acute respiratory syndrome* (SARS), and the causative agent was designated as the SARS-associated CoV (SARS-CoV). No vaccine or antiviral agent was available to fight the pandemic, which was ultimately ended through intense public health intervention, including massive screening programs, voluntary quarantine, and travel restrictions. This human infection apparently started as a CoV that jumped from its natural animal host, possibly a civet cat, to humans. SARS-CoV highlights the public health risk that can occur when animal viruses suddenly appear in a susceptible human population.

In 2012, a SARS-like virus was linked to severe respiratory tract infections in the Middle East. This virus was named the Middle East respiratory syndrome CoV (MERS-CoV). By August 2013, MERS-CoV was associated with 94 infections and 46 deaths, primarily in Saudi Arabia. MERS-CoV is unlike any other CoV that infects humans. In 2014, another outbreak of MERS-CoV occurred in Saudi Arabia, with 14 people infected and 4 reported deaths. As of July 2022, over 2600 cases and 858 confirmed deaths of MERS-CoV infection in 27 countries have been reported.

The 2002 SARS outbreak created much interest in understanding the epidemiology, reservoir-host relationship, and vaccination possibilities associated with this CoV. In 2007, SARS-CoV antibodies were detected in 47 of 705 South African and Democratic Republic of the Congo bat sera samples collected from 1986 to 1999. Researchers tested bats from the Rocky Mountain region in 2006 and detected CoV RNA in two species of bats: *Eptesicus fuscus* and *Myotis occultus*. Animals, such as civets, can acquire infections with SARS-like CoV from contact with infected bats. Current data indicate that bats, primarily horseshoe bats, are the most likely reservoir for SARS-CoV, although the bat CoVs are species specific. More than 10 mammalian species have been identified as being susceptible to SARS-CoV by natural or experimental infection. Infections of these secondary hosts may give rise to strains that could potentially infect humans.

On December 31, 2019, the existence of patients with pneumonia of an unknown etiology was reported to the WHO by national authorities in China. It was first seen in Wuhan, China, and was thought to have emerged in a food market. This virus was officially identified by the coronavirus study group as SARS coronavirus 2 (SARS-CoV-2), and the resulting outbreak of a coronavirus-associated acute respiratory disease was named coronavirus disease 19 (COVID-19).

From China, COVID-19 rapidly spread into Europe. The first reported case in the United States was on January 21, 2020, in Washington state in a 35-year-old male patient who had just returned from Wuhan, China. On February 20, 2020, the CDC announced that the first person in the United States had died from COVID-19. By July 2022, the WHO reported that the COVID-19 pandemic resulted in over 577,000,000 confirmed cases and over 6,400,000 deaths worldwide. For the same time period, the CDC reported over 90,000,000 U.S. cases with over 1,000,000 deaths.

This virus is highly infectious, and although most individuals are asymptomatic or have mild symptoms, those with certain medical conditions often progress to severe, life-threatening illness. High-risk conditions include diabetes, overweight or obesity, history of smoking or cancer, and heart and kidney disease. Severe illness is linked to two primary causes: secondary pneumonia and an overexuberant proinflammatory cytokine response. This cytokine response is referred to as a *cytokine storm* or *hyperinflammation*. Cytokine storm syndrome is an uncontrolled activation and amplification of the host immune response resulting in the release of several cytokines such as tumor necrosis factor, interleukin-1 (IL-1), IL-6, and IL-18. The cytokine response appears to be related to the severity of ARDS, which is a type of respiratory failure characterized by widespread inflammation in the lungs.

SARS-CoV targets the epithelial cells of the respiratory tract and is transmitted from person to person by direct contact, droplet, or airborne routes. Other organ systems affected by SARS include the spleen, lymph nodes, digestive tract, urogenital tract, CNS, bone marrow, and heart. The virus can also be isolated from urine and feces, suggesting other potential routes of transmission. The receptor for SARS-CoV-2 is angiotensin-converting enzyme 2. This enzyme is expressed on many human cells including those in the respiratory airways, intestine, kidney, heart, and pancreas. Certain individuals may be genetically more susceptible to SARS than others.

CoVs are extremely fragile and difficult to culture, but it is

Case check 29.1

History of leukemia and hypogammaglobulinemia and advanced age placed the patient in the Case in Point at high risk for severe COVID-19. Fortunately, the two treatments of SARS-CoV-2 convalescent plasma he received helped him to remain asymptomatic. However, he was culture positive for the virus several weeks after his diagnosis.

possible to detect viral antigens from some species directly by IF or ELISA (EUROIMMUN Medical Laboratory Diagnostics, Lübeck, Germany). Viral particles can also be detected by electron microscopy in stool samples. However, sensitivity is generally lower with electron microscopy and antigen detection than RT-PCR. The most common diagnostic approach for the identification of CoVs is amplification and detection of virus-specific RNA using RT-PCR. RT-PCR assays have demonstrated greater sensitivity and specificity and a much shorter TAT. Many viral panels are now available for the detection of the four endemic CoVs found in the United States—CoV 229E, CoV HKU1, CoV NL63, and CoV OC43—from respiratory specimens (FilmArray, BioFire Diagnostics). Other nucleic acid-based tests include isothermal amplification methods and loop-mediated amplification assays. These are typically used in research or larger reference laboratories. Antibodies can be detected through western blot analysis and ELISA. Although serologic assays have largely been replaced by NAATs, they still have a role in epidemiologic studies. The COVID-19 pandemic has resulted in an expansion of testing for CoV-2. However, at time of publication, all testing for detection and exposure has EUO authorization from the FDA and CDC.

The treatment of COVID-19 has been based on providing basic life support and mitigating the effects on the respiratory

system caused by ARDS, which is the leading cause of mortality. Modulating cytokine levels is key in preventing widespread organ damage, respiratory failure, and death. Several drugs with antiinflammatory activity in other illnesses were evaluated in respect to their potential utility in the treatment of the hyperinflammation induced by SARS-CoV-2 infection, and these included interferon, steroids, atazanavir, favipiravir, lopinavir-ritonavir, anakinra, baricitinib, and remdesivir. The current preferred therapy in most cases is ritonavir-boosted nirmatrelvir (Paxlovid). The anti-inflammatory agent dexamethasone is used in severe cases. Heparin is added in cases with coagulopathy.

Multiple COVID-19 vaccines were developed, and two successful vaccines were approved for EUO authorization in December 2020. These vaccines are unique in that they use messenger RNA (mRNA) coding for a viral protein. After injection, cells in the recipient use the mRNA to express the viral spike protein, which initiates the immune response to the virus. Although these vaccines are new, mRNA vaccine technology began in the 1980s.

Filoviridae

The family Filoviridae includes eight genera; *Marburgvirus* and *Ebolavirus* are the most clinically significant. The genus *Marburgvirus* contains one species (*Marburg marburgvirus*) and two viruses: Marburg virus (MARV) and Ravn virus. The genus *Ebolavirus* contains five viruses: EBOV, Sudan virus (SUDV), Reston virus (RESTV), Tai Forest virus, and Bundibugyo virus. Both Marburg virus and EBOV share a common morphology (Fig. 29.21) and similar genome and structural proteins. These viruses have many similarities; they rarely cause human infections, they cause infections with high mortality rates, and they have unknown reservoirs in nature, although human infection can result from contact with infected monkeys.

Lake Victoria Marburg virus, now known as MARV, was named after the location of one of the first outbreaks: Marburg, Germany. In 1967, 32 people from Marburg and Frankfurt, in Germany, and from Belgrade, Serbia, and Yugoslavia contracted an unknown infection, and 7 died. Epidemiologists noted that the deceased individuals all worked in vaccine-producing facilities and had contact with African green monkeys



Fig. 29.21 Transmission electron micrograph of an Ebola virus (×100,000). (Courtesy Fred A. Murphy, Centers for Disease Control and Prevention, Atlanta, GA.)

that had arrived recently from Uganda. The virus had been transmitted to 24 other people through healthcare-associated transmission, casual contact, and sexual contact. Patients with secondary infection had a milder illness, and all survived the infection. Outbreaks of MARV are rare.

The EBOVs are named after the Ebola River in the Democratic Republic of the Congo, where the infection first emerged in 1976. The virus emerged almost simultaneously in Sudan. In Zaire, a patient treated at a village hospital for a bloody nose probably introduced the virus into the hospital, where it was then transmitted nosocomially and via contact with individuals at home. Nuns in the hospital routinely reused syringes without sterilizing them. Therefore the hospital amplified the number of cases. Infections were also passed to the victims' families, often during a process in which the intestines of infected deceased males were cleansed to prepare the bodies for funerals.

The two simultaneous outbreaks of Ebola fever were caused by two different viruses, Ebola Zaire, now called *Ebola virus*, and Ebola Sudan, now called *Sudan virus*. Of the two, EBOV is the more virulent. In the initial outbreak, 318 individuals were infected in Zaire; the mortality rate was 88%. In the Sudan outbreak, 284 people were infected, and a mortality rate of 53% was reported. After the initial two large outbreaks, smaller outbreaks occurred. In Sudan, 34 cases occurred in 1979, and 65% of the 34 patients died. The virus seemed to retreat into the jungles for the next 15 years.

An unusual sequence of events occurred in 1989, when monkeys imported to Reston, Virginia, from the Philippines were afflicted by an epidemic of infection by what eventually became the third type of EBOV, RESTV. Four workers in the animal facility developed antibodies to RESTV but did not develop the disease. RESTV was isolated from the bloodstream of one of the workers. EBOV continued to emerge periodically in the Democratic Republic of the Congo (2003 and 2007), southern Sudan (2004), and Uganda (2007). In 2012, several more outbreaks were reported. Beginning in March 2014, West Africa had the largest outbreak of EBOV in history. Multiple countries were involved, with Liberia, Sierra Leone, and Guinea affected the most through early 2016. According to the CDC, there were 28,652 probable cases, with 15,261 laboratory confirmed cases and 11,325 deaths during this outbreak. This outbreak resulted in precautions in both Europe and the United States to handle suspected cases of infected people traveling from West Africa. Many clinical laboratories have now established protocols to handle suspected cases, ensuring safely for both staff and other patients. Newer molecular testing is being developed to detect EBOV from serum, and this will likely obtain rapid FDA review and approval.

Symptoms of EBOV and MARV disease are similar, producing a hemorrhagic fever. After an incubation period of 4 to 16 days, there is sudden onset of fever, chills, myalgia, and anorexia. Patients then develop a sore throat, abdominal pain, diarrhea, vomiting, and a maculopapular rash, and they begin to bleed from injection sites and the GI tract. Hemorrhaging in skin and the internal organs may occur as well. Diagnosis of the infection can be made using RT-PCR, IF, or viral culture. However, because of the risk of laboratory infection, biosafety level 3 laboratories are required. Sometimes electron microscopy of clinical samples will yield the characteristic long, rodlike virions.

Flaviviridae

The family Flaviviridae contains several important human pathogens, many of which are zoonotic arboviruses, including Japanese encephalitis virus, dengue virus, yellow fever virus, St. Louis encephalitis (SLE) virus, Zika virus, Kyasanur Forest disease virus, Langat virus, Löping ill virus, Murray Valley encephalitis virus, Omsk hemorrhagic fever virus, Powassan virus, tickborne encephalitis virus, Wesselsbron virus, and WNV. Japanese encephalitis virus is a major cause of encephalitis in Asia and is the most common cause of arboviral encephalitis in the world. Because it is being reported in regions previously free from Japanese encephalitis, including Australia, Japanese encephalitis virus is considered an emerging pathogen. Currently, nearly 68,000 cases of Japanese encephalitis are reported annually. Because most patients are asymptomatic, cases are likely under-reported. Disease ranges from a flulike illness to acute encephalitis. Children are mostly affected by this infection, with mortality as high as 30%; mortality in adults is much lower.

Another important member of the family Flaviviridae is dengue virus, which causes two distinct diseases: classic dengue fever (DF) and dengue hemorrhagic fever (DHF). Worldwide, tens of millions of cases of DF and approximately 500,000 cases of the more serious DHF occur annually. The average mortality associated with DHF is 5%, accounting for 22,000 deaths each year. Most deaths occur in children younger than 15 years. The virus is transmitted by *Aedes* mosquitoes, including *Aedes aegypti* and *Aedes albopictus*. These mosquitoes infest more than 100 countries and bring the risk of DF to 2.5 billion people.

Dengue virus has four serotypes (1 to 4); DF is a relatively mild infection. Patients with DF develop fever, headache, myalgia, and bone pain (resulting in the nickname *break-bone fever*). Some patients also develop a rash. The disease is self-limiting and often resolves in 1 to 2 weeks. Although classic DF is a mild disease, DHF is not. Patients develop DHF after previous exposure to one serotype of dengue virus and are then exposed to one of the other three serotypes. Exposure to two different serotypes of dengue virus appears to be necessary for development of DHF. Patients with DHF develop the symptoms of classic DF, along with thrombocytopenia, hemorrhage, shock, and sometimes death.

Yellow fever, caused by yellow fever virus, is also considered an emerging infection. Although a safe vaccine has been available for decades, about 200,000 cases of yellow fever with 30,000 resulting deaths are reported annually worldwide. The actual incidence may greatly exceed these numbers. The emergence results from increased spread of the mosquito vectors, deforestation of Africa and South America, and increased travel to endemic regions. The vaccine has greatly reduced or eliminated the transmission of yellow fever in some countries. However, yellow fever is still epidemic in parts of Africa and South America, where about 80% of the population must be vaccinated to reduce the impact of the disease.

Patients bitten by mosquitoes carrying yellow fever virus can develop an asymptomatic or acute infection involving fever, myalgia, backache, headache, anorexia, nausea, and vomiting. Most patients experiencing acute disease recover after about 4 days. However, about 15% enter a systemic toxic phase in which fever reappears. The patient develops jaundice (hence the name *yellow fever*) as well as bleeding from the mouth, eyes, nose, stomach, or other areas. The kidneys

may fail, and about 50% of patients in the toxic phase die. The other 50% recover without serious sequelae.

The three different transmission cycles for yellow fever virus are sylvatic, urban, and intermediate cycles. In the sylvatic cycle, yellow fever virus is maintained in monkey populations and transmitted by mosquitoes. Monkeys become sufficiently viremic to pass the virus to mosquitoes as they feed on the monkeys, thus keeping the transmission cycle active. Humans are not the usual hosts when they enter jungle areas in which the sylvatic cycle exists.

The urban cycle occurs in larger towns and cities when infected *A. aegypti* mosquitoes transmit the infection to humans. Because infected humans can continue the transmission when bitten by uninfected mosquitoes, large outbreaks can occur from a single case of yellow fever. The intermediate transmission cycle of yellow fever occurs in smaller villages in Africa. In the intermediate cycle, humans and monkeys are reservoirs, while mosquitoes serve as reservoirs and vectors in the high-morbidity, low-mortality outbreaks. In the intermediate cycle, mosquitoes can transmit yellow fever virus from monkeys to humans, and vice versa. If patients who develop yellow fever from the intermediate cycle travel to larger cities and are bitten by mosquitoes, they can trigger an outbreak of urban yellow fever.

From 2011 to 2020, a total of 103 SLE neuroinvasive disease cases were reported in the United States. Patients with SLE are most likely to be asymptomatic; hence total cases are much higher. While cases are seen from Canada to Argentina, most human cases occur in the United States. Symptomatic patients may develop a fever only, whereas some patients develop meningoencephalitis. The mortality rate of symptomatic patients is 3% to 20%. Unlike many of the arboviral infections, SLE is milder in children than in adults; older patients have the greatest risk of serious illness and death. SLE is transmitted to humans by the bird biting *Culex* mosquitoes. Most infections occur in the summer months.

First isolated and identified in 1937 from a febrile patient in the West Nile district of Uganda, WNV is an ssRNA virus and member of the Japanese encephalitis antigenic complex, like SLE. The virus is transmitted by a mosquito vector between birds and humans. The virus replicates actively inside the avian host; however, the virus does not replicate well in humans, making humans a dead-end host for infection. The incidence of WNV infection in the United States increased during the mid-1990s, prompting the development of a national surveillance system in 1999. In 2003, the CDC documented 9862 human cases of WNV infection in 44 states, the most ever reported in one year. A decline followed in 2011 to 712, the lowest number of cases ever reported. The number of cases has fluctuated since then. In 2019, a total of 971 cases were reported, and 633 were described as neuroinvasive. A total of 60 patients (9%) with neuroinvasive disease died. From 1999 to 2019, overall, about 16% of the cases were in California and 13% in Texas.

Approximately 80% of individuals infected with WNV are asymptomatic. The remaining 20% display symptoms of what is termed *West Nile fever*, which includes fever, headache, fatigue, occasional rash on the trunk, swollen lymph glands, and/or eye pain. The primary risk factor for serious neuroinvasive disease is age greater than 50 years. Neuroinvasive disease typically manifests as meningitis or encephalitis.

Laboratory tests approved for the detection of WNV include IgG and IgM ELISA, including a rapid WNV ELISA assay and an indirect IF assay for antibodies. WNV ELISA assays can cross-react with other flaviviruses and should be confirmed by antibody neutralization. IgM antibody does not cross the blood-brain barrier; therefore the presence of IgM in CSF strongly suggests CNS infection. WNV can be present in tissues, blood, serum, and CSF of infected humans or animals. Several RT-PCR, TaqMan, and nucleic acid sequence-based amplification assays have been used for confirmation of WNV infection. There is no specific treatment for WNV infection, but in severe cases requiring hospitalization, supportive care, including IV fluids, respiratory support, and prevention of secondary infection, may be warranted.

Zika virus is an insect vector-borne disease that is most commonly transmitted through *Aedes* (*A. aegypti* and *A. albopictus*) mosquitoes. Zika virus can also be transmitted by exposure to infected blood or sexual contact. Less commonly, Zika virus can be transmitted from a mother to a child during pregnancy. Zika virus is typically endemic to parts of Africa and Asia. However, in 2015 to 2016, an epidemic occurred across South, Central, and North America, where the disease was previously unreported. Almost 500,000 cases of Zika virus infection were reported in 38 countries throughout the Americas. Almost 50,000 of these cases were laboratory confirmed, and 360,000 cases were suspected to be positive. In 2016, the CDC reported more than 5000 cases of Zika virus infection, with 95% of the cases in travelers returning from affected areas. Reported cases have dropped significantly since then, with only 4 cases found in the United States and 57 cases in U.S. territories in 2020.

Symptoms of Zika virus infection in most people resemble those of infections with other arboviruses, such as chikungunya virus (primarily fever, headache, and fatigue). The epidemic in Brazil between 2015 and 2016, resulting in over 300,000 cases, was marked by the detection of the virus in fetal amniotic fluid and an increased reporting of cases of *microcephaly* (small head size) in newborns.

Orthomyxoviridae

The influenza viruses are enveloped and belong to the family Orthomyxoviridae. These viruses are distinguished by using two major structural proteins: matrix protein (M) and nucleoprotein (NP). This places the influenza viruses into four genera within the family: *Influenzavirus A*, *Influenzavirus B*, *Influenzavirus C*, and *Influenzavirus D*. Influenza viruses have a worldwide distribution and originate as zoonotic infections, carried by several different species of birds and mammals. The influenza season in the southern hemisphere is from May to October and in the northern hemisphere is from November to April.

Influenza A virus remains one of the most crucial health problems worldwide. In the pandemic of 1918 and 1919, influenza infected an estimated 500 million people, about one third of the world's population, and killed at least 50 million people, including more than 600,000 in the United States. However, since that event, the world has only been able to react to the threat of influenza, rather than vanquish it. A typical influenza season in the United States hospitalizes almost 700,000 and kills about 36,000 people.

Influenza viruses are enveloped; types A and B have eight segments of ssRNA, while types C and D have seven. Influenza A viruses are classified into subtypes using the two major surface glycoproteins: hemagglutinin (H) and neuraminidase (N). The H antigen is used to bind to host cells, and the N antigen cleaves budding viruses from infected cells. The H protein is the major antigenic determinant detected by antisera. There are 17 H antigens (H1 to H17), although human infections usually occur only with H1, H2, and H3. There are 10 N antigens (N1 to N10); human infections usually occur with N1 and N2.

The key to the persistence of influenza virus is its antigenic variation. Each year, antigenic drifts are caused by RNA replication errors of the virus. **Antigenic drift** is a minor change in antigenic structure as mutations accumulate. Antigenic drift occurs with all four influenza viruses—A, B, C, and D. The surface antigens sometimes can change drastically, causing an **antigenic shift**, resulting in a new H or N antigen. Antigenic shift is associated only with influenza A virus. There are two mechanisms of antigenic shift. The first is genetic reassortment of the eight ssRNA strands of two separate influenza strains. Pigs have receptors for both avian and human influenza viruses as well as swine influenza viruses and can be co-infected with all three types of viruses. Reassortment occurs when the genomes of different influenza viruses combine into a single virion, resulting in a new strain of influenza virus. The second mechanism is an adaptive mutation, in which a novel virus slowly adjusts and becomes transmissible from a mammalian (including human) host. Shifts result in novel strains of influenza virus, so the human population is likely to have little or no historic exposure or resistance to the new strain, which greatly increases the risk of pandemics.

Three major shifts occurred during the 20th century. Infection with influenza A virus (H1N1), the *Spanish flu*, appeared in 1918 to 1919. H1N1 was the predominant strain until a shift to influenza A virus (H2N2) occurred in 1957 to 1958. This shift resulted in the *Asian flu* pandemic. In 1968, another shift occurred, and a pandemic strain of influenza A virus (H3N2) resulted in the *Hong Kong flu*. The dominant strains of influenza A virus since 1977 have been influenza A viruses H1N1 and H3N2.

In 1998, an outbreak of infection with influenza A virus (H5N1), the *avian flu*, appeared in poultry in Hong Kong. At least 18 humans acquired the disease via contact with birds that year, and 6 deaths were reported. H5N1 is a highly pathogenic avian influenza (HPAI) virus; historically, most human infections are acquired after direct or close contact with infected birds. The H5N1 influenza virus appeared in flocks in parts of Asia in 2004 and again in 2005, resulting in large poultry losses in Japan, Cambodia, China, Vietnam, and nearby countries. Some humans were infected during these poultry outbreaks. Again, the incidence of human disease was low, but the mortality rate was high, although it is possible that some mild cases went undiagnosed.

Studies documented wide dissemination of influenza A virus (H5N1) throughout Asia because of migrating birds. By early 2006, influenza A virus (H5N1) had been isolated from birds in Turkey, Greece, Italy, Germany, Iran, Iraq, Nigeria, and many other countries. In April 2013, a novel avian flu caused by influenza A virus (H7N9) was reported in China. Only a few cases were reported initially, but 5 of the 11 human cases were fatal. Human-to-human transmission was not

documented. The potential for the avian viruses to adapt to the human host, through genetic reassortment or adaptive mutation, remains an important concern for future influenza seasons.

In the spring of 2009, a highly infectious novel form of influenza, *swine flu*, caused by influenza A virus (H1N1) emerged. It was first reported throughout Mexico and forced school closings and cancellation of sporting events. By October 2009, the WHO had received reports of more than 375,000 human cases and 4525 deaths. Because many countries stopped counting individual cases, the actual number was much larger. The outbreak quickly reached phase 6, which is the WHO definition of a pandemic. This was the first influenza pandemic in 40 years. In April 2010, the WHO announced that the H1N1 influenza virus had moved into the post-pandemic period. H1N1 was found to contain RNA from avian, human, and porcine strains of the virus.

In 2011, human infections caused by influenza A H3N2v, a nonhuman influenza virus variant normally found in swine, were reported. When viruses that normally circulate in animals infect humans, they are termed *variant viruses*. This H3N2v virus was first detected in January 2011, and it had genes from avian, porcine, and human viruses and the 2009 H1N1 pandemic virus M gene.

Because the H and N antigens of influenza A virus continually change, the CDC and the WHO make recommendations for the composition of the quadrivalent influenza vaccine several months before the influenza season begins. The current U.S. vaccine usually contains two different influenza A virus strains, H1N1 and H3N2, and two influenza B virus strains. Influenza B virus infections, which also can occur seasonally, are usually less common than influenza A virus infections, although epidemics of influenza B virus infections can occur every few years. It is highly recommended that persons with reduced immune status (e.g., those with HIV, those with diabetes, and older adults) be vaccinated annually with the inactivated virus vaccine to prevent infection, because it can be rapidly fatal in these populations. A pediatric version for children 6 to 35 months is available. The use of an attenuated quadrivalent intranasal vaccine is only recommended for individuals between 2 and 49 years of age who are in good health.

Influenza C virus can cause mild upper respiratory tract illness in humans, indistinguishable from the common cold. Its genome consists of seven ssRNA segments, lacking the gene coding for neuraminidase, as in influenza A and B viruses. Studies have shown influenza C virus to be more stable genetically compared with influenza A virus, and although reassortment does occur in the former, it is less prone to major changes in infectivity. The influenza D virus was isolated from a pig in Oklahoma in 2011. Subsequently, it has been isolated from other animals. Little is known about its likelihood to cause human infections.

Influenza viruses are spread through aerosol inhalation. Infection may also be spread by handling fomites. Viruses attack the ciliated epithelial cells lining the respiratory tract, causing necrosis and sloughing of the cells. The incubation period is 1 to 4 days. Although asymptomatic infections can occur, infections are usually characterized by cough, sore throat, rapid onset of malaise, fever, chills, myalgia, and often a nonproductive cough. The fever can be as high as 41° C (105.8° F). Infected patients are ill for as long as 7 days, and

convalescence may require more than 2 weeks. Influenza can also cause a fatal viral pneumonia. Complications include secondary bacterial pneumonia.

The best specimens for diagnosis are nasopharyngeal swabs, washes, or aspirates collected early in the course of the disease. Flocked swabs (Copan Diagnostics, Corona, CA) are reported to collect significantly more epithelial cells from the nasopharynx compared with rayon swabs. Specimens should never be frozen. Several rapid kits are commercially available for the diagnosis of influenza in about 30 minutes. Some of these kits are of low complexity and are waived by the CLIA. Some kits can detect and distinguish between influenza A and B viruses, but some cannot distinguish between them or detect only influenza A virus. These tests are valuable in outpatient settings, and their low cost and ease of use make them ideal for quick screening. Influenza virus can also be identified in respiratory secretions by using DFA, EIA, and immunochromatographic assays.

Nucleic acid–based assays are also used for the detection of influenza viruses, with the most common method being RT-PCR. These assays are fast becoming the method of choice for detection. Some can be completed in less than 30 minutes, have a low cost, and are easy to perform. They can be used as point-of-care tests, thus improving patient care.

Influenza viruses grow in the amniotic cavity of embryonated chicken eggs and various mammalian cell culture lines, such as PMK and MDCK cells. Influenza-infected culture cells adsorb RBCs, a feature that can be used to detect positive cell cultures. Positive cultures can be detected rapidly using IF staining of infected monolayers grown in shell vials or flat-bottomed wells of microtiter plates.

The antiviral drugs amantadine and rimantadine can prevent infection or reduce the severity of symptoms if administered within 48 hours of onset of infection. These antiviral drugs are effective only against influenza A virus. A newer class of antivirals, termed *neuraminidase inhibitors*, is available. These agents, such as zanamivir (Relenza) and oseltamivir (Tamiflu), are more expensive compared with amantadine but provide coverage against infections by influenza A virus and influenza B virus. Current CDC guidelines highly recommend both zanamivir and oseltamivir as primary treatments in confirmed cases of influenza. There has been significant resistance to amantadine by influenza A virus (H3N2), so it is not recommended for treatment of infection by this virus. There have been some reports of resistance to oseltamivir by influenza A virus (H1N1), but use of this agent is still recommended as a primary treatment.

Paramyxoviridae

Parainfluenza viruses

Several genera belong to the family Paramyxoviridae, including *Morbillivirus*, and *Rubulavirus*. Four types (1 to 4) of PIVs cause disease in humans. Human PIV-1 and PIV-3 belong to the genus *Respirovirus*; PIV-2 and PIV-4 belong to the genus *Rubulavirus*. PIVs are enveloped helical RNA viruses with two surface antigens: hemagglutinin-neuraminidase (HN) antigen and fusion (F) antigen. HN antigen is the viral adhesion molecule; F antigen is responsible for the fusion of the virus to the cell and of one infected cell to another infected cell.

PIV-1 infections occur most often in the fall every other year, and the incidence of PIV-2 is generally lower than that of

PIV-1 and PIV-3. PIV-2 is seen every 2 years alternating with PIV-1. PIV-3 occurs almost every year in spring and summer and can be seen yearlong in temperate climates. PIV-4 is seldom isolated, but routine testing is not readily available.

PIVs are a primary cause of respiratory disease in young children. PIV-1 and PIV-2 cause the most serious illnesses in children between 2 and 4 years of age. PIV-1 is the primary cause of croup (laryngotracheobronchitis) in children. PIV-3 causes bronchiolitis and pneumonia in infants and is second in importance only to RSV. PIV-4 generally causes mild upper respiratory tract infections. The viruses are spread through respiratory secretions, aerosol inhalation, and direct contact. Infection of the cells in the respiratory tract leads to cell death and an inflammatory reaction in the upper and lower portions of the respiratory tract. Rhinitis, pharyngitis, laryngotracheitis, tracheobronchitis, bronchiolitis, and pneumonia can result.

Direct examination of nasopharyngeal secretions by IF can give rapid results. Some newer nucleic acid assay panels now include PIV-1, PIV-2, and PIV-3, which aid in treatment and epidemiology efforts. Most of these newer PCR technologies are on panels that include several other respiratory pathogens. Serologic assays are more valuable for epidemiologic studies than for diagnostic purposes. The best specimens for viral culture are aspirated secretions and nasopharyngeal washes. Specimens for viral isolation should be taken as early in the illness as possible, kept cold, and inoculated into PMK cells or LLC-MK2 cells. The viruses can be identified by using hemadsorption, IF, or EIA techniques. Aerosolized ribavirin can be used to treat infection. No vaccines are available, and infection control measures like those for RSV are used to prevent spread in health care facilities.

Mumps virus

Mumps virus is related to PIVs and is in the genus *Rubulavirus*. It is an enveloped virus with HN and F surface antigens. Mumps virus, which has a global distribution, is spread through droplets of infected saliva. It causes an acute illness producing unilateral or bilateral swelling of the parotid glands, although other sites, such as the testes, ovaries, and pancreas, can be infected. The virus infects primarily children and adolescents and usually results in long-lasting immunity. The primary infection of the ductal epithelial cells in the glands results in cell death and inflammation.

A vaccine effective in controlling the disease is available. Two doses are recommended for better immunity—the first at 12 to 15 months and the second at 4, 6, 11, or 13 years of age. The annual incidence of mumps in the United States varies. From 2000 to 2020, cases ranged from about 200 to over 6000. Only 154 cases were reported in 2021. Large multistate outbreaks of over 6000 mumps cases were documented in 2006, 2016, and 2017. The reasons for the outbreaks remain largely unknown. They have occurred in communities of individuals who received one or two vaccines. Outbreaks are generally seen in close-contact settings. During the outbreak of 2016 to 2017, most cases (nearly 3000) occurred in a close-knit community in northwest Arkansas. From 2009 to 2010, another outbreak, with 3502 cases in a New York camp, was reported and seemed to confirm waning immune protection by the vaccine.

The mumps virus can be isolated from infected saliva and swabbing of the Stensen duct, from 9 days before onset of symptoms until 8 days after parotitis appears. The virus, which is relatively fragile, can also be recovered from urine

and CSF. Specimens may be examined directly by using IF and EIA methods. Studies have shown viral isolation using shell vial cultures of Vero or LLC-MK2 cells to be more successful than those with HEp2 or HeLa cell lines. Isolates can be identified by hemadsorption inhibition, IF, and EIA tests.

Paired sera can be tested for mumps antibody by EIA, IF, and hemagglutination inhibition tests. Paired sera taken at as small an interval as 4 to 5 days can demonstrate a diagnostic or fourfold increase in titer. Cross-reactions between soluble and viral antigens can confuse the interpretation of serologic results. Virus isolation is preferable, although primary health care providers rarely have trouble recognizing mumps clinically.

Measles virus

The measles virus is an enveloped virus classified in the genus *Morbillivirus*. It is found worldwide; in temperate zones, epidemics occur during winter and spring. At one time, measles (rubeola) was the most common viral disease in children in the United States. An average of 500,000 cases of measles were reported annually in the 1950s, with an average of 500 deaths. Immunization programs began in the United States in 1963, and the reported number of cases decreased to fewer than 1500 by 1983. A reemergence of measles occurred in 1989 to 1991, attributed to immigration and lack of vaccination. The decision to administer a second dose of vaccine to school-age children has drastically reduced the incidence of measles in the United States. From 2010 to 2021, the number of cases has ranged from 13 to 667. The WHO reported a 50% surge in measles cases from 2016 to 2019, with 869,770 total cases and 207,500 deaths. This is the highest number of reported cases since 1996.

Measles is highly contagious and spreads by aerosol. Initial replication takes place in the mucosal cells of the respiratory tract. The measles virus then replicates in the local lymph nodes and spreads systemically. The virus circulates in T and B cells and monocytes until eventually the lungs, gut, bile duct, bladder, skin, and lymphatic organs are involved. After an incubation period of 7 to 10 days, there is an abrupt onset, with symptoms of sneezing, runny nose and cough, red eyes, and rapidly increasing temperature. About 2 to 3 days later, a maculopapular rash appears on the head and trunk. **Koplik spots**, lesions on the oral mucosa consisting of irregular red spots with a bluish-white speck in the center (Fig. 29.22),

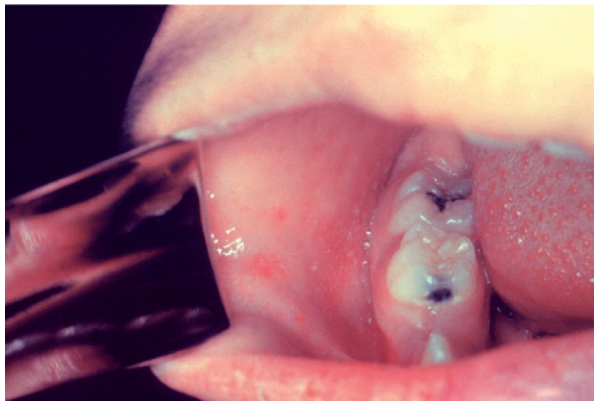


Fig. 29.22 Patient presenting on the third pre-eruptive day with Koplik spots indicative of the onset of measles. (Courtesy Centers for Disease Control and Prevention, Atlanta, GA.)

generally appear 2 to 3 days before the rash and are diagnostic. Complications such as otitis, pneumonia, and encephalitis may occur. A progressive, highly fatal form of encephalitis can occur but is rare. In developing countries with malnutrition and poor hygiene, measles can have a high fatality rate. Infection confers life-long immunity. An effective attenuated vaccine is available and recommended for all children.

Measles is easily diagnosed clinically, so few requests for laboratory identification are made. The virus is fragile and must be handled carefully. The specimens of choice are from the nasopharynx and urine, but the virus can be recovered from these sources only in the early stages of infection. The virus grows in PMK cells, causing the formation of distinctive spindle-shaped or multinucleated cells. Virus isolates can be identified by using serum neutralization, EIA, or IF tests. Serologic diagnosis of measles is accomplished by demonstrating measles-specific IgM in the specimens collected during the acute phase of the disease. Nucleic acid testing should be considered for diagnostic use if IgM testing is compromised by the recent use of measles virus-containing vaccine as part of a routine vaccination or in response to a suspected outbreak.

Respiratory syncytial virus

RSV, a member of the genus *Orthopneumovirus*, causes croup, bronchitis, bronchiolitis, and interstitial pneumonia. It is the most common cause of severe lower respiratory tract disease among infants and young children worldwide. Almost one half of all infants are infected by RSV during their first year of life, and almost all have been exposed to RSV by 2 years of age.

The CDC estimates 2.1 million outpatient visits among children younger than 5 years of age, 58,000 hospitalizations, and 14,000 deaths annually. Because infection does not confer complete immunity, multiple infections can occur throughout life and can be severe in older adults, the immunocompromised, and those with cardiac and respiratory problems. It is estimated that 24 of 1000 children with RSV infection will be hospitalized. For this reason, healthcare-associated RSV is a problem in many medical facilities. Recommendations to reduce the risk of healthcare-associated spread include testing hospital personnel and infants with upper respiratory tract infections for RSV, isolating infants with RSV infection, following good handwashing and personal protective equipment practices, limiting visitation, and organizing patients and staff members into cohorts.

RSV can be a significant cause of morbidity and death in older patients. With a rapidly growing aging population, RSV in elder care facilities is becoming a significant problem in the United States. Unlike the bronchiolitis caused by RSV in children, pneumonia often develops in adults. The virus spreads mostly through large-particle droplets and contact with fomites rather than through inhalation of small aerosols. The virus may be carried in the nares of asymptomatic adults. RSV infections occur in yearly outbreaks that last 2 to 5 months and usually appear during winter or early spring in temperate zones.

RSV can be identified in specimens from nasopharyngeal swabs and washes by using DFA or EIA. Rapid antigen detection kits are also available for RSV, but they have a relatively low sensitivity. Several NAATs are available for RSV testing, with most taking less than 1 hour to provide results. These are normally part of a multiplex assay for other respiratory

pathogens. Because the virus is extremely fragile, recovering it from cultures is difficult, but culture remains the gold standard. It is important to collect samples within the first few days of illness. In about 50% of cases, antigen detection and viral cultures are negative at 1 week. Specimens for culture must be kept cold but cannot be frozen. RSV grows readily in continuous epithelial cell lines, such as HEp2, forming syncytia. It also grows in PMK and human diploid fetal cells. Once the CPE is detected, RSV can be identified by using IF, EIA, and serum neutralization tests.

The antiviral compound ribavirin is approved for treatment of RSV infection. However, some controversy regarding the efficacy of ribavirin therapy still exists. A monoclonal antibody, palivizumab (PVZ), which blocks RSV entry into the host cell, is also approved for RSV treatment and prophylaxis in high-risk children. Often, ribavirin is recommended to be used in combination with PVZ. No vaccine for RSV infection is available.

Human metapneumovirus

Human metapneumovirus (HMPV) was first described in children with previously virus-negative cultures in 2001. Infected children display many clinical symptoms that mimic infections with RSV, influenza virus, and PIV. Often, these diseases are ruled out early in diagnosis, which leads to a presumptive identification of HMPV. Serologic and RNA sequence studies have shown that the virus is found worldwide in all age groups. Most children have been exposed by the time they reach 5 years of age. One half of the lower respiratory tract infections seen in children in the first 6 months of life are caused by this virus. Clinical disease ranges from mild upper respiratory tract infection to acute lower respiratory tract infection and includes fever, nonproductive cough, sore throat, wheezing, congestion, shortness of breath, and lethargy. HMPV infections usually occur in the winter months, but outbreaks have been documented during summer.

HMPV infections usually occur in children. A survey in Finland among 1338 children younger than 13 years of age found that 47 (3.5%) with respiratory illness were positive for HMPV. The highest concentration of illness (7.6%) was seen in children younger than 2 years of age. Co-infections with another virus, including enterovirus, rhinovirus, influenza virus, and PIV, were detected in eight (17%) of the infected children. HMPV has also been documented to cause outbreaks in long-term care facilities. In the summer of 2006, HMPV affected 26 residents and 13 staff members in a 171-bed California long-term care facility. All affected residents had an underlying medical condition; two were hospitalized, but none died. This outbreak indicates a year-round risk of infection in institutionalized older adults. Treatment for HMPV infection is mostly supportive.

Specimens for detecting HMPV can be collected from the nasopharynx by using flocked swabs placed in transport media and transported to the laboratory for culture or molecular analysis. Specimens should be processed immediately but can be stored at 4°C for up to 72 hours. DFA and RT-PCR assays are used for identification, although DFA has lower sensitivity. The respiratory viral panel assay from Luminex Molecular Diagnostics (Toronto, Canada) claims 100% sensitivity and 98.2% specificity for identification of HMPV in clinical specimens. HMPV grows slowly in standard cell culture lines, such as monkey kidney and A549 cell lines.

BOX 29.1 Genera within the family Picornaviridae of human significance

Genus <i>Aphthovirus</i>
Foot and mouth disease virus
Genus <i>Enterovirus</i>
Enteroviruses species A to D (about 106 types)
Polioviruses types 1 to 3
Echoviruses
Human coxsackieviruses
Rhinovirus species A to C (about 165 types)
Genus <i>Parechovirus</i>
<i>Parechovirus</i> species A and B (about 24 types)
Genus <i>Hepatovirus</i>
Hepatitis A virus

Picornaviridae

Picornaviridae is one of the largest families of viruses, with more than 280 members. It contains many important human and animal pathogens. Four genera with human clinical significance belong to the family Picornaviridae: *Aphthovirus*, *Enterovirus*, *Hepatovirus*, and *Parechovirus* (see Box 29.1). The genus *Hepatovirus* includes HAV. This virus is discussed in detail in the “Hepatitis Viruses” section later in this chapter.

Enteroviruses

These small naked viruses cause various conditions, including fever of unknown origin, aseptic meningitis, paralysis, sepsis-like illness, myopericarditis, pleurodynia, conjunctivitis, exanthemas, pharyngitis, and pneumonia. Enteroviruses have also been implicated in early-onset diabetes, cardiomyopathy, and fetal malformations.

Most serotypes of the enteroviruses are distributed worldwide. In temperate zones, enterovirus epidemics occur in summer and early fall. Enterovirus infections are more prevalent in areas with poverty, overcrowding, poor hygiene, and poor sanitation. Viruses are spread via aerosol inhalation, the fecal-oral route, and fomites. The portal of entry is the alimentary canal via the mouth. The viruses replicate initially in the lymphoid tissues of the pharynx and gut. Viremia can result in the virus spreading from these locations to the spinal cord, heart, and skin. The clinical disease caused by enteroviruses can be neurologic, respiratory, or cardiac, depending on viral spreading and the immune status of the host. Enterovirus infections most often cause mild nausea and diarrhea in adults. However, disease can be much more severe in neonates because of their immature immune system.

The polioviruses tend to infect the CNS and cause paralysis in a small percentage of infected individuals. The viruses destroy their host cells. In the intestines, damage is temporary because the cells lining the gut are rapidly replaced. In contrast, neurons are not replaced, which results in neuron death and permanent paralysis.

No vaccines are available for enteroviruses other than poliovirus. Good personal and health care facility hygiene and proper sanitation can reduce the incidence of enterovirus infections. Poliovirus vaccines of attenuated or inactivated viruses are available. Since 1988, the polio vaccine program has been crucial to the WHO's effort to eradicate polio worldwide. In countries where polio is considered to have endemic rates of incidence, there has been a steady decrease since

the program began. In 1988, 125 countries reported endemic rates; by 1999, the number of such countries had decreased to 30, and in 2013 to only 3: Afghanistan, Nigeria, and Pakistan. In 2020, only two countries were considered endemic for wild-type poliovirus: Afghanistan and Pakistan. Unfortunately, interruptions in vaccine programs has caused an increase in cases. The WHO reported 175 cases in 2019, up from only 33 in 2018; transmission continues to be widespread in Pakistan. In 2020, there were 1107 cases of circulating vaccine-derived poliovirus infections; 443 were in the two endemic countries, and 664 were in nonendemic countries.

Enteroviruses can be cultured from pharyngeal specimens immediately before the onset of symptoms and for 1 to 2 weeks afterward; the viruses can be isolated from feces for as long as 6 weeks thereafter. However, ideally, specimens should be obtained early in the course of the infection. Specimens from the throat, feces, rectum, CSF, and conjunctiva are recommended.

Polioviruses, type B coxsackieviruses, and echoviruses grow readily in several cell lines, including PMK, continuous human and primate, and human fetal diploid fibroblast lines. The high-numbered enteroviruses (68 to 71) require special handling. The CPE appears quickly and is readily identifiable. Enteroviruses have no group antigen, so they must be identified individually by a serum neutralization test. The WHO distributes pools of enterovirus antisera that allow identification by neutralization patterns in the antisera. The CPE and resistance to detergent, acid, and solvents constitute a presumptive diagnosis of enterovirus infection.

Hand, foot, and mouth disease (HFMD) is caused primarily by coxsackievirus types A5, A10, and A16 and occasionally by enterovirus type 71. HFMD generally occurs in young children. Outbreaks of HFMD caused by EV71 have been reported, mostly in children in East and Southeast Asia, with some cases seen in Cambodia and China, resulting in the death of hundreds. It is spread by fomites or via the oral-fecal route. A mild prodromal phase can develop, with malaise, headache, and abdominal pain. Small, painful sores suddenly appear on the tongue, buccal mucosa, and soft palate. Simultaneously, a maculopapular rash appears on the hands, feet, and buttocks, followed by bullae on the soles of feet and the palms of hands. The lesions regress in about 1 week. If a rash develops, it is transient. The virus can be isolated from specimens from swabs of the mouth and bullae. Coxsackievirus A16 grows in PMK and human diploid fibroblast cells and can be identified by serum neutralization tests.

More than 150 serotypes of rhinoviruses exist, and they are the major cause of the common cold. Most people experience two to five colds each year, and almost 50% of these colds are caused by the rhinoviruses. Rhinovirus infections occur throughout the year, but their incidence increases in winter and spring. Transmission is primarily via aerosols, but contact with secretions and fomites can also cause infection.

Rhinoviruses infect the nasal epithelial cells and activate the mediators of inflammation. Symptoms include a profuse watery discharge, nasal congestion, sneezing, headache, sore throat, and cough. In severe cases, bronchitis and asthma can result. Unfortunately, no cure for the common cold has been found. Treating symptoms and reducing the spread of the virus in the household is the typical response. Both natural and recombinant interferons have been shown to be effective in preventing infection and illness when given intranasally over short periods. However, prolonged administration has resulted in adverse effects, such as nasal irritation, ulceration, and bleeding.

Case in point

A 36-year-old man was admitted to the hospital after presenting at the emergency department with a self-reported, 7-month history of numbness and weakness in his right leg. He had lost 25 lb, was experiencing bowel incontinence, and had been unable to urinate for 3 days. Two years previously, the patient had been diagnosed with HIV infection. A physical examination demonstrated bilateral lower extremity weakness, and his reflexes were slowed throughout his body. KS lesions were noted, especially on the lower extremities, along with thrush and herpes lesions in the perianal region. The patient had no fever, and magnetic resonance imaging ruled out spinal cord compression. The patient had a history of intravenous drug abuse, chronic diarrhea for 1.5 years, KS for 2 years, and pancytopenia for several weeks. The patient had large right arachnoid cysts of congenital origin. No previous laboratory report indicated infectious agents in the CSF. Meningitis was suspected, and the patient was admitted with a diagnosis of polyradiculopathy (neuropathy of the spinal nerve roots) secondary to AIDS. Blood and CSF were collected. Although numerous white blood cells were found, the CSF produced no growth on routine bacteriologic culture. The blood cultures were also negative. Acyclovir was administered after culture results were received.

Retroviridae

The family Retroviridae contains several subfamilies, including Oncovirinae and Lentivirinae. The retroviruses have a unique mode of replication; they require an RNA-dependent DNA polymerase (reverse transcriptase) to synthesize DNA from the RNA genome. The human T-lymphotropic viruses HTLV-1, HTLV-2, and HTLV-5 belong to the subfamily Oncovirinae. These viruses are not cytolytic but are associated with several leukemias, sarcomas, and lymphomas.

HIV belongs to the subfamily Lentivirinae. Although some groups of individuals, mostly in West Africa, are infected by HIV-2, it is the impact of HIV-1 that continues to be felt around the world. HIV causes AIDS. HIV-1 was identified first and is responsible for the AIDS pandemic. The WHO estimates about 37.7 million people were living with HIV/AIDS at the end of 2020 and approximately 1.5 million new cases were diagnosed. Approximately 680,000 HIV/AIDS-related deaths occurred in 2020. The region most severely affected by HIV/AIDS is sub-Saharan Africa, which has approximately 25.4 million patients with HIV infection, accounting for 67% of the total worldwide incidence. Children share this burden; in 2020, approximately 1.7 million children younger than 15 years of age had HIV/AIDS, and about 150,000 cases of newly infected children were reported.

It was reported that at the end of 2019, an estimated 1.2 million people 13 years of age and older had HIV/AIDS in the United States, and there were 36,801 new HIV cases. Approximately 14% are not aware of their infection. Of the new cases, approximately 81% were men. The greatest number of persons who contract HIV/AIDS are MSM. This group accounted for 70% of the new cases.

The virus is transmitted via blood and exchange of other body fluids. HIV is cell associated, so fewer viruses are found in cell-free plasma than in whole blood, and even fewer viruses are found in saliva, tears, urine, or breast milk. HIV is not highly contagious, and normal, social, nonsexual contact

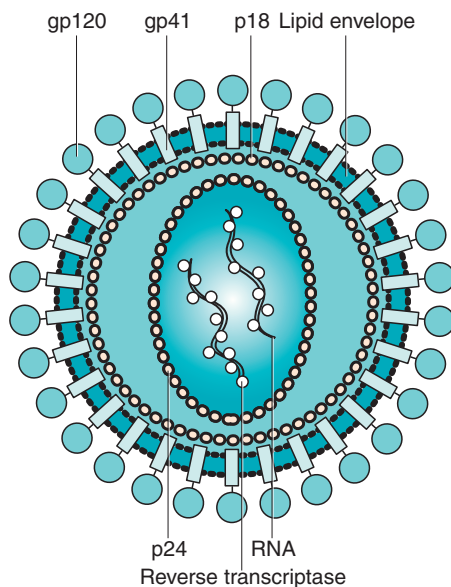


Fig. 29.23 Human immunodeficiency virus (HIV).

poses no threat to individuals. High risk for contracting the virus includes unprotected sex with multiple partners, IV drug abuse, infusion of blood and blood products, and presence of the virus in a pregnant woman who passes it to the fetus during pregnancy. Individuals with ulcerative sexually transmitted infections (STIs), e.g., syphilis, genital herpes, chancroid, are at greatest risk. Donor blood and blood products are screened for HIV, which substantially reduces the risk.

HIV is a spherical virus, with a three-layer structure (Fig. 29.23). In the center are two identical copies of ssRNA and reverse transcriptase surrounded by an icosahedral capsid. The nucleocapsid is enclosed by a matrix shell to which an envelope of host cell origin is attached. Inserted into the viral envelope are viral glycoprotein (gp) trimers or spikes. The diagnostically important HIV antigens are the structural proteins p24, gp41, gp120, and gp160.

Once HIV enters the body, the primary target cells are the CD4⁺ T cells, monocytes, and macrophages. Acute infections are generally mild and can resemble infectious mononucleosis. The individual will then enter a period of clinical latency, and even though the virus is replicating rapidly in lymphoid tissues, the virus is not detectable in the bloodstream, and the patient remains asymptomatic. Eventually lymphopenia results, with the greatest loss in the CD4⁺ T-cell population. Healthy individuals have CD4⁺ T-cell counts of at least 1000/mm³, whereas patients with HIV/AIDS can have counts lower than 200/mm³. Lymph nodes become enlarged and hyperplastic. The virus destroys the T-helper cells, which are critical in host immune response to infectious agents. The patient begins experiencing several chronic and recurrent infections (Box 29.2). As the disease progresses, the CD4⁺ cell count continues to decline, and the severity of opportunistic infections increases. The patient can also develop virus-induced cancers, such as KS. Death usually occurs because of opportunistic infections, although HIV-1 itself can directly cause encephalitis and dementia.

In adult patients living in developed countries, the average length of time from HIV infection to development of AIDS is about 10 years. About 20% develop AIDS within 5 years, and fewer than 5% have an asymptomatic HIV infection for

BOX 29.2 Common opportunistic infections and cancers in patients with acquired immunodeficiency syndrome

- Candidiasis of the respiratory tract
- Coccidiomycosis
- Cryptococcal meningitis
- Cryptosporidiosis with persistent diarrhea
- Cytomegalovirus infections of organs other than the liver, spleen, or lymph nodes
- Histoplasmosis
- Persistent herpes simplex virus infections including the respiratory tract
- Kaposi sarcoma or lymphoma of the brain in patients older than 60 years of age
- Oral hairy leukoplakia
- Lymphoid interstitial pneumonia, pulmonary lymphoid hyperplasia, or both in children younger than 13 years of age
- Invasive cervical cancer
- *Mycobacterium avium* complex, *Mycobacterium kansasii* infection
- *Pneumocystis jirovecii* pneumonia
- Progressive multifocal leukoencephalopathy
- Toxoplasmosis of the brain in infants younger than 1 month of age
- Wasting disease

Case check 29.2

Clinical manifestations of HIV infection include CNS involvement, opportunistic infections, and tumors. CNS involvement is often seen in HIV-associated dementia complex but can manifest itself as other neurologic problems, such as loss of bowel or bladder control and weakness in the extremities. KS develops from the cells lining the lymph or blood vessels. The resulting lesions are red, purple, or brown blotches or tumors on skin. Other than their appearance, the skin lesions of KS often cause no symptoms. However, in some cases, they may cause painful swelling and be more painful when found in the legs, groin, or skin around the eyes. KS in such sites as the liver, lungs, or digestive tract may be life-threatening because of abnormal bleeding or difficulty breathing.

periods longer than 10 years. The rate at which the virus multiplies in the host is related to the onset of AIDS. This rate can be measured with an HIV viral load assay, a quantitative gene amplification technique that measures the amount of HIV-1 RNA in the plasma.

Laboratory diagnosis of HIV infection is generally based on demonstration of anti-HIV antibodies and, in some cases, detection of viral antigens and RNA. Serologic tests suffer from the disadvantage of the *window period*, the time between infection and the ability to detect antibodies (seroconversion). HIV antibodies are normally produced within a few weeks after infection. The window period varies depending on the assay, but the fourth-generation assays have a median window period of 18 days. Several assays are commercially available as screening tests using different methods, including EIA and IF.

The early diagnostic kits, referred to as *first-generation screening tests*, used purified viral lysate as antigens. The second-generation tests used recombinant viral proteins, thus improving performance. The third-generation tests relied on the double-antigen sandwich assay. In this procedure, viral

antigen attached to a solid-phase bound antibody to HIV from the patient's serum. Labeled HIV antigen was then added, captured by the patient's antibody, and measured. Fourth-generation kits detect antibody and HIV-1 p24 antigen. The first fifth-generation assay is a multiplexed screening test that detects and differentiates three HIV analyte markers: anti-HIV-1 antibodies, anti-HIV-2 antibodies, and the p24 antigen. The immunoassays are generally based on EIA or chemiluminescent immunoassay methods. By detecting antigen, early infections could be identified before antibody is produced. These newest assays are considered the standard of care for diagnosis of HIV infection.

Several rapid assays screen for HIV infection by using serum, plasma, and saliva. By 2012, the FDA had approved six rapid tests for the diagnosis of HIV infection: OraQuick Advance rapid HIV-1/2 antibody test (OraSure, Bethlehem, PA); Uni-Gold Recombigen HIV test (Trinity Biotech, Wicklow, Ireland); Reveal G2 rapid HIV-1 antibody test (MedMira, Halifax, Canada); Multispot HIV-1/2 rapid test (Bio-Rad Laboratories, Hercules, CA); and Clearview HIV-1/2 and Clearview Complete HIV-1/2 (Inverness Medical Professional Diagnostics, Princeton, NJ). OraQuick for whole-blood and oral fluid specimens, Clearview Complete for whole blood, and Uni-Gold for whole-blood samples have waivers from the CLIA. Currently, the FDA has approved one home collection kit, the Home Access HIV-1 test system (Home Access Health Corporation, Maria Stein, OH). In 2012, the first FDA-approved in-home HIV test kit was the OraQuick In-home HIV Test (OraSure Technologies), and by 2016, the FDA had approved multiple tests in each category.

Current CDC guidelines (published in 2014 and revised in 2018) recommend using a screening test that detects HIV-1 and HIV-2 antibodies and p24 antigen. No further testing is required if the test result is negative. Any reactive result on a screening test should be tested with a supplemental antibody immunoassay that differentiates HIV-1 antibodies from HIV-2 antibodies. If both tests are reactive, the specimen should be interpreted as positive for HIV. A specimen positive in the initial screening test and negative in the supplemental test should be tested with a NAAT. A positive NAAT indicates an acute HIV infection. A specimen with a negative NAAT should be referred for testing with a different validated supplemental HIV-2 test (antibody test or NAAT) or repeat testing in 2 to 4 weeks. Other immunologic markers of HIV infection are listed in [Box 29.3](#).

The western blot has been the reference method for HIV diagnosis for many years. Viruses grown in cell cultures are lysed, and the proteins are separated by electrophoresis and transferred to nitrocellulose. Antibodies to several HIV proteins and glycoproteins can be detected. For a positive result, CDC guidelines require detection of at least two antibodies

to the three proteins p24, gp41, and gp120/160. Western blot results should also be followed by tests for anti-HIV-2 antibodies ([Fig. 29.24](#)).

Because these viruses readily develop resistance to drugs, HIV infection is often treated with combination therapy. Highly active antiretroviral therapy (HAART) involves aggressive combination therapy soon after HIV infection is diagnosed, ideally within 7 days. In hospital exposures, a similar strategy is used if a health care worker is accidentally exposed to the virus. Aggressive therapy is initiated after the exposure, and this significantly reduces the risk of contracting an infection. Despite aggressive therapy, a cure is not achieved. In 2008, scientists reported that an American living in Berlin (*the Berlin patient*) was cured of HIV infection. The patient received a bone marrow transplant from a donor with a CCR5 mutation (CCR5 Δ 32). CCR5 is a coreceptor for HIV adhesion. The mutation prevents virus infection. Subsequently, two additional patients receiving similar treatment were also reported to be cured, the most recent in 2022. Additional reports of cures in patients receiving HAART have been made; however, these have not been substantiated.

Several classes of antiviral drugs are approved for treatment. Nucleoside reverse transcriptase inhibitors (NRTIs) were the first class of retroviral drugs developed and are incorporated into viral DNA; these include adefovir, azidothymidine, dideoxyinosine, d4T (stavudine), 3TC (lamivudine), and tenofovir. NRTIs inhibit the conversion of nucleoside analogues in the body to nucleotide analogues. Non-NRTIs attach to reverse transcriptase, preventing conversion of RNA to DNA; examples include delavirdine, nevirapine, and

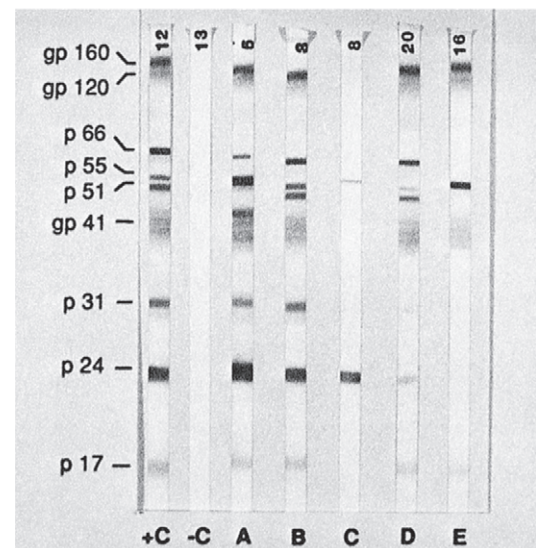


Fig. 29.24 Human immunodeficiency virus immunoblot. Reactive protein (p) and glycoprotein (gp) bands appear as purplish lines across the strip. Proteins with higher molecular weights appear at the top of the strip. Structural and nonstructural proteins are given RNA structural genome codes: GAG for group-specific antigens; POL for polymerase; and ENV for envelope. ENV codes for glycoprotein precursors: gp160, gp120, and gp41 to gp43. POL codes for p65, p51, and p31. GAG codes for p55, p24, and p17. Results are negative, indeterminate, or positive based on the pattern on the strip. Positive corresponds to reactivity to two or more of the following antigens: p24, gp41, or gp120/gp160. Indeterminate corresponds to the appearance of one or more bands in a pattern that does not satisfy the positive criteria. Negative corresponds to the absence of any band on the strips. (Courtesy Patricia A. Cruse.)

BOX 29.3 Important immunologic markers for acquired immunodeficiency syndrome

- Steady decline in number of CD4⁺ T cells
- Depression of the CD4⁺-to-CD8⁺ cell ratio to less than 0.9 (reference value, ≥ 1.5)
- Functional impairment of monocytes and macrophages
- Decreased natural killer cell activity
- Anergy to recall antigens in skin tests

efavirenz. Other classes of antiviral drugs include protease inhibitors (e.g., ritonavir, saquinavir, indinavir, amprenavir), fusion inhibitors (e.g., enfuvirtide), integrase strand transfer inhibitors (e.g., dolutegravir, elvitegravir, raltegravir), and chemokine receptor antagonists (e.g., maraviroc). HAART includes combinations, such as two NRTIs combined with a protease inhibitor.

HIV viral load assays can predict therapeutic efficacy. In different studies, suppression of HIV RNA levels to less than 5000 copies of RNA/mL for up to 2 years was correlated with an increase in CD4⁺ cell counts, up to 90/mm³. In contrast, patients with HIV RNA loads of more than 5000 copies of RNA/mL generally showed a decrease in CD4⁺ cell counts. Usually, these assays are performed monthly so therapy can be adjusted on an individual basis.

Case check 29.3

In HIV infection, the immune system is severely compromised, giving rise to many opportunistic infections caused by viruses, bacteria, fungi, and protozoa, as well as neoplastic disease, wasting syndrome, progressive multifocal leukoencephalopathy, and HIV encephalopathy. These complications often result in death. Meningitis occurs frequently in patients with HIV infection and can be caused by a variety of agents. In the Case in Point, the patient was treated with acyclovir for a presumed HSV meningitis and/or encephalitis. Acyclovir is an inactive nucleoside analogue that is metabolized by viral thymidine kinase into an active form, which results in premature termination of DNA during synthesis.

Rhabdoviridae

Rabies is caused by several strains of viruses belonging to the genus *Lyssavirus* in the family Rhabdoviridae. An estimated 55,000 persons die as a result of rabies worldwide annually, with 40% of these being children younger than 15 years of age. Dogs are implicated in 99% of rabies deaths among humans. In the United States, human cases of rabies are rare, with approximately two per year. However, rabies is an emerging infection in animals, with all mammals being susceptible. Most rabies infections 40 years ago occurred in dogs, with some occurring in cats, foxes, and skunks. However, in the United States, rabies is now more closely associated with wild animals (92%), such as raccoons, skunks, foxes, and bats. Programs to vaccinate domestic animals have reduced the number of rabies cases in dogs and cats, which has, in turn, decreased the risk to humans.

Humans usually acquire the rabies virus when they are bitten or scratched by animals with rabies. With the number of endemic areas increasing among wildlife, the risk of human exposure to rabies increases because of the increased likelihood of encountering a wild animal with rabies or a domestic animal that has contracted rabies from wildlife. Humans infected with the rabies virus experience a brief prodromal period of pain at the exposure site and have vague flulike symptoms. Mental status changes, such as anxiety, irritability, and depression, can also occur. After the prodromal period, patients suffer additional CNS changes, including hallucinations, paralysis, excessive salivation, hydrophobia, bouts of terror, seizures, respiratory and cardiac abnormalities, and hypertension. These symptoms are followed ultimately by

coma and death. In 2004, a Texas hospital encountered five cases of rabies. This occurrence was a result of transplanting organ and tissues from a person who was later discovered to have been a victim of a bat bite into four patients, all of whom subsequently died because of the disease. Rabies virus is not considered a bloodborne pathogen; therefore it is likely that the spread was caused by infected nerve tissue that came with the new organs.

Laboratory diagnosis of rabies involves determining whether an animal that has bitten a human has rabies. The animal is killed, and its head is removed and sent to a reference laboratory. The fastest and most sensitive method of identifying rabies virus in a specimen is by using a direct IF technique. Impression smears should be made from various areas of the brain, primarily the hippocampus, pons, cerebellum, and medulla oblongata. In living patients suspected of having rabies, skin biopsy, especially at the hairline at the back of the neck, and an impression of the cornea may be performed. The presence of rabies virus in these specimens is diagnostic, but its absence merely means that no virus is present in those specific specimens, not that the patient does not have rabies. EIAs are currently the most sensitive assays to use for serologic tests. Rabies virus can be grown in suckling or young adult mice, murine neuroblastoma, or related cell lines.

Rabies cannot be successfully treated once symptoms appear. However, postexposure prophylaxis is 100% effective in preventing the disease if the bite victim is treated immediately after exposure. Postexposure prophylaxis includes vigorously cleansing the wound site, providing human anti-rabies immunoglobulin, and administering a three-injection series of the rabies vaccine. Each year, more than 15 million people worldwide receive postexposure preventive treatment. This is estimated to prevent 327,000 rabies deaths annually.

Two approved human vaccine preparations are available in the United States: IMOVAX (Sanofi Pasteur Biologics, Canton, MA) and RabAvert (GlaxoSmithKline, Philadelphia, PA). The vaccine can be given to persons who may have been exposed to rabies, such as veterinarians, laboratory personnel, people who explore caves, and those visiting high-risk countries for longer than 30 days. Only one unvaccinated person with rabies has ever survived. A female teenager in Wisconsin developed rabies about 1 month after being bitten by a rabid bat. She was put into a coma and treated with several antiviral compounds. However, the reason for her survival is still not completely understood. Her survival resulted in a new method of rabies treatment called the Milwaukee Protocol.

Togaviridae

The family Togaviridae contains the genera *Alphavirus*, *Rubivirus*, and *Arterivirus*. No member of the genus *Arterivirus* is known to infect humans. Many of the viruses in the genus *Alphavirus* are mosquito-borne and cause encephalitis. Eastern equine encephalitis (EEE), which causes disease in horses and humans, occurs primarily in the eastern half of the United States. From 2010 to 2020, 120 human cases were reported, with a mortality rate of about 45%. Infections can cause a range of effects, from mild flulike symptoms to encephalitis. Of those who survive, almost 50% suffer permanent CNS

damage. Birds are the natural reservoirs of the virus, which is spread to humans and horses via bites of mosquitoes. Because horses and humans are dead-end hosts, EEE in horses can be a predictor of human EEE cases.

The western equine encephalitis (WEE) virus also causes disease in humans and horses. WEE virus causes a milder disease compared with EEE virus, and patients develop an asymptomatic or mild infection consisting of fever, headache, nausea, and mental status changes. Of young children and infants who survive, 5% to 30% will suffer permanent CNS damage. Mortality is about 3%. Since 1964, 639 cases were reported in the United States, but none have been reported since 1994.

Venezuelan equine encephalitis (VEE) has caused large outbreaks of human and equine encephalitis in the Americas. Outbreaks of epizootic strains have affected up to 75,000 people during a single epidemic. Death is much less common in patients with VEE than in patients with WEE or EEE. Between 4% and 14% of infected individuals will develop neurologic disease. Infected adults often develop a flulike illness, whereas encephalitis is more commonly seen in children with VEE.

Rubella virus is an enveloped virus belonging to the genus *Rubivirus*. It causes the disease rubella, or German measles, a mild febrile illness accompanied by an erythematous, maculopapular, discrete rash with postauricular and suboccipital lymphadenopathy. Like measles, rubella occurs most frequently in winter and spring. The diseases are so similar that as many as 50% of suspected measles cases are diagnosed as rubella. The rubella virus is transmitted via droplets. The virus is present in nasopharyngeal secretions or any secretion of infected infants, who shed the virus in large amounts for long periods. A rash starts on the face and spreads to the trunk and limbs. No rash appears on the palms and soles. About 50% of those infected with rubella virus are asymptomatic. Transient polyarthralgia and polyarthritis can occur in children and are common in adults.

Rubella would be of little concern if it did not cross the placenta and spread to the fetus, which results in congenital rubella syndrome. The syndrome can cause effects ranging from birth of a normal infant to birth of a severely impaired infant, fetal death, or spontaneous abortion. Because the

rubella virus halts or slows cell growth, the impact on the embryo is worse when the infection develops in the earliest stages of pregnancy.

An effective attenuated vaccine is available and should be administered to all children and to young women before they become sexually active. In 2004, the CDC declared the United States rubella free, meaning an absence of continuous disease transmission for 12 months or more. Currently, fewer than 10 cases are reported annually, and since 2012, all cases were acquired outside the country. Before the vaccination program began in 1969, about 12.5 million people contracted rubella annually.

It is important to collect specimens (e.g., throat swabs) the day the rash appears. Direct examination of specimens with RT-PCR has been attempted. However, not all are significantly sensitive to be used on clinical specimens, and no protocol for detecting viral RNA has been established. Several molecular tests are being evaluated. Isolation procedures are difficult because of the lack of a cell line that exhibits cytopathic effect. Infected cells can be detected by RT-PCR. Serologic procedures are effective because any rubella antibody is presumed to be protective. The most sensitive serologic assays are the solid-phase and passive hemagglutination tests. Latex agglutination and antigen-coated RBC tests are useful but less sensitive.

Hepatitis viruses

The hepatitis viruses are grouped together, not because of their structural or genetic similarities but because they share the same tissue tropism—the liver. Before the 1970s, patients with hepatitis were classified as having infectious hepatitis or serum hepatitis. Infectious hepatitis was transmitted from person to person via the fecal-oral route, and serum hepatitis resulted from transfusion of or exposure to infected blood and blood products. During the past several years eight different human hepatitis viruses have been recognized. The most clinically significant are HAV, HBV, HCV, and hepatitis D virus (HDV) (see [Table 29.7](#)). Four additional viruses have been described: hepatitis E virus (HEV), hepatitis G virus (HGV), SEN virus, and transfusion-transmitted virus (TTV).

Table 29.7 Clinical and epidemiologic differences among HAV, HBV, HCV, and HDV

Clinical Features	Hepatitis A (HAV)	Hepatitis B (HBV)	Hepatitis C (HCV)	Hepatitis D (HDV)
Incubation (days)	15–45	30–120	40–50	21–90
Type of onset	Acute	Insidious	Insidious	Usually acute
Mode of transmission				
Fecal-oral	Usual	Not likely	Not likely	Not likely
Parenteral	Increasing	Usual	Likely	Usual
Other	Foodborne, waterborne	Intimate contact, trans-mucosal transfer	Vertical transmissionIntranasal cocaine use	Intimate contact, less efficient than for hepatitis B virus
Sequelae				
Carrier	No	Yes	Yes	Yes
Chronic hepatitis	No	Yes	Yes	Yes
Mortality (%)	0.1–0.2	0.5–2.0	0.2–0.3	30 (chronic form)

HAV and HEV are transmitted via the fecal-oral route; HBV, HCV, HDV, HGV, SEN virus, and TTV are transmitted via contact with infected blood and blood products. HBV, TTV, and SEN virus have DNA genomes, whereas the others have an RNA genome.

Despite the biological and morphologic differences among the hepatitis viruses, many of the clinical symptoms caused by them are similar. Therefore differentiation based on clinical findings should not be relied on for diagnosis. The most common signs and symptoms are fatigue, headache, anorexia, nausea, vomiting, abdominal pain (right upper quadrant or diffuse), jaundice, and dark-colored urine.

Hepatitis A virus

HAV is a small, icosahedral, naked ssRNA virus, the sole member of the genus *Hepatovirus* in the family Picornaviridae. HAV infects people of all ages. In the United States, reported cases declined from the 1990s, when the annual average was about 25,000, to 2014, when 1239 cases were reported. In 2015, cases began increasing and reached 18,846 in 2019. The 30- to 39-year age group had the highest rate of infection, 14.5/100,000 population. In 2022, the CDC reported an outbreak of HAV infection linked to fresh organic strawberries. As of June 2022, 18 cases were confirmed. The WHO estimates that 1.5 million clinical cases of HAV infection occur each year.

HAV is almost always transmitted via the fecal-oral route and is usually acquired through close personal contact or via contaminated food. The risk factors for HAV infection include sexual or household contact with an infected person, daycare contacts, foodborne or waterborne outbreaks, IV drug use, and international travel. However, almost 50% of the cases in the United States have no established risk factor. The virus is shed in large amounts in feces during the incubation period and early prodromal stage, and food and water contamination

can result. The incubation period for HAV infection is approximately 1 month. After infection, individuals experience a transient viremia, after which the virus reaches the liver and replicates in hepatocytes. The virus passes into the intestine, and viral shedding begins and can persist for months.

Infections in more than 90% of children younger than 5 years of age tend to be asymptomatic. In adults, symptoms can range from mild to severe prolonged hepatitis. The onset is abrupt, and patients experience fever, chills, fatigue, malaise, aches, pains, and, in some cases, jaundice. The infection is self-limiting, with convalescence possibly lasting weeks. Complete recovery can take months. HAV infection has a low mortality rate and no persistence and does not cause chronic liver damage.

The most common method for laboratory diagnosis of HAV infection is to demonstrate IgM to HAV (Fig. 29.25). Isolation of HAV is not practical because it is difficult to grow in culture and tends to mutate drastically. Some reference laboratories offer in-house RT-PCR assays that can detect HAV infection before liver enzymes are elevated. Studies comparing antibody detection with RT-PCR have shown that HAV RNA can be detected much earlier after infection. Vaccination of children has the potential to reduce the incidence of HAV infection. Other vaccination target groups include people who travel to countries with endemic HAV, MSM, drug abusers, and patients with chronic liver disease. Persons who have not been vaccinated and have been exposed to HAV can receive immunoglobulin therapy, which is 80% to 90% effective in preventing infection when administered soon after exposure. Immunoglobulin therapy can also be used as preexposure prophylaxis.

Hepatitis B virus

HBV is an enveloped, partially dsDNA virus that belongs to the family Hepadnaviridae. The virus contains the hepatitis B surface antigen (HBsAg), which circulates in the bloodstream

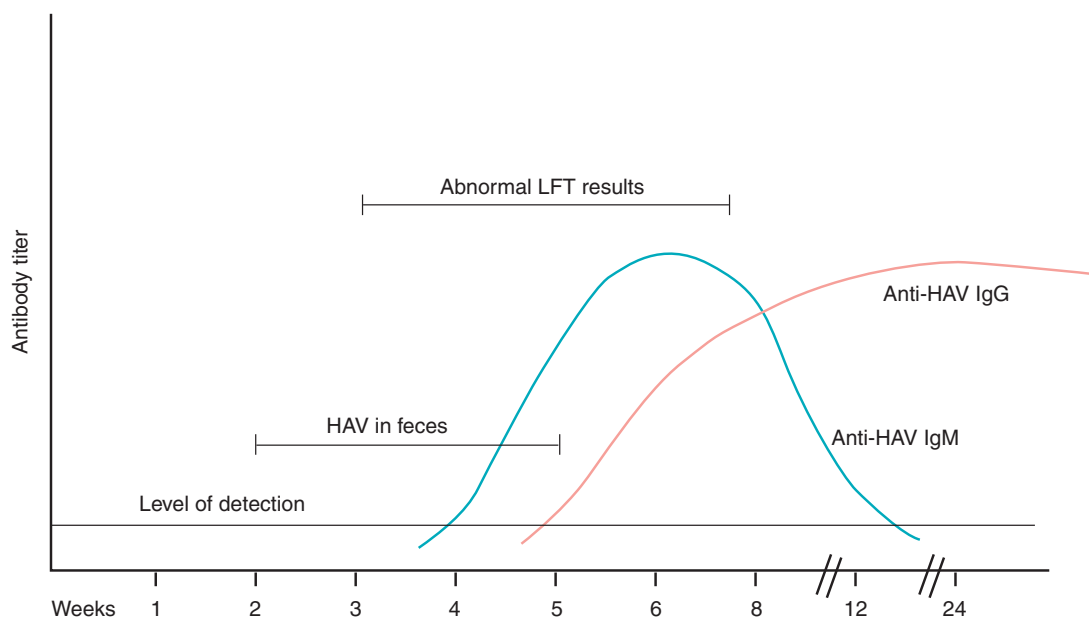


Fig. 29.25 Serologic evaluation of hepatitis A virus (HAV) infection showing the increase and decrease of detectable antibodies. *IgG*, Immunoglobulin G; *IgM*, immunoglobulin M; *LFT*, liver function test.

as 22-nm particles. The whole virus has a total diameter of about 45 nm. The virion also contains a hepatitis B core antigen (HBcAg) and hepatitis B envelope antigen (HBeAg). Eight genotypes of HBV have been identified (A to H), and several studies have shown a difference in clinical outcome based on the genotype.

Almost one half the world's population lives in areas with endemic HBV, and more than 8% of the population is positive for HBsAg. According to the WHO, about 296 million people worldwide are infected with HBV. In the United States, the prevalence rate among adults decreased from 5.7/100,000 in 1999 to 1.0 in 2019. The incidence of acute HBV infection is estimated to be about 20,700 cases annually.

HBV is primarily a bloodborne pathogen. Infected individuals can have as many as 1 million infectious particles per milliliter of blood. Lower concentrations of virus appear in semen, vaginal fluid, and saliva. Many other body fluids (e.g., tears, urine, sweat, breast milk) contain HBsAg but do not seem to be infective. The main modes of transmission are through sexual, perinatal, and parenteral routes. In the United States, heterosexual and male homosexual contacts are the most common routes of transmission. High-risk groups include IV drug abusers, MSM, individuals from endemic areas, persons with household or sexual contacts with HBV carriers, health care personnel, people with tattoos or body piercings, and infants born to HBV-positive mothers. Almost one third of the patients who become infected, however, have no known risk factor.

The human cost of infection is high. An estimated 820,000 deaths per year worldwide are related to HBV infection. Once HBV enters the host, it travels from the bloodstream to the liver and infects the hepatocytes. Cytotoxic T cells then attack the HBV-infected hepatocytes. The incubation period for HBV infection ranges from 2 to 6 months, with an insidious onset that includes symptoms of fever, anorexia, and hepatic tenderness. Jaundice occurs in only about 10% of children who are younger than 5 years of age and is much more common in older children and adults (32% to 54%).

As the immune response is activated, the virus is slowly cleared from the system, and most patients become noninfectious. In adults, about 50% of infections are asymptomatic; 20% to 30% of patients exhibit clinical jaundice but have a benign resolution of the infection. Therefore about 80% of infections do not cause serious sequelae. The risk for chronic infection is inversely proportional to the age at the time of infection, with approximately 90% of infants and only 3% of adults developing chronic infection. Individuals with chronic infection have a higher risk of liver disease, such as cirrhosis or hepatic carcinoma. A safe and effective recombinant vaccine is available for preventing HBV infection. The U.S. Advisory Committee on Immunization Practices recommends that the series begins at birth and be completed by 6 to 18 months.

Diagnosis of HBV infection is based on clinical presentation and demonstration of specific serologic markers for HBV (Box 29.4). Serum aminotransferase levels also increase in infected patients. The presence of HBsAg in a patient's serum indicates that the patient has an active HBV infection, is a long-term carrier, or is in an incubation period. IgM anti-HBc appears early in the course of the disease and indicates an acute infection. In patients in whom HBsAg is not detected and anti-HBs has not yet appeared, detection of IgM anti-HBc confirms the diagnosis of acute HBV infection. The period

BOX 29.4 Serologic markers for the diagnosis of hepatitis B virus infection

- HBsAg—hepatitis B surface antigen, the envelope protein consisting of three polypeptides
- Anti-HBs—antibody to hepatitis B surface antigen
- Anti-HBc—antibody to hepatitis B core antigen
- HBeAg—antigen associated with the nucleocapsid, also found as soluble protein in serum
- Anti-HBe—antibody to hepatitis B envelope antigen

between the inability to detect HBsAg and the detection of anti-HBs antibodies is referred to as the *core window*. The detection of anti-HBs antibodies in serum indicates convalescence or immune status.

When the infection resolves, IgG anti-HBc and anti-HBs antibodies become detectable in the patient's serum. The presence of HBsAg after 6 months of acute infection is a strong indication that the patient is a long-term carrier; the appearance of HBeAg in this case is indicative of a chronic infection and high infectivity. Table 29.8 shows the interpretation of HBV serologic markers. Fig. 29.26 depicts the increase and decrease in the levels of detectable serologic markers during acute HBV infection and resolution and presentation of chronic HBV infection. Several molecular assays are available that detect viral DNA and provide a short TAT and very sensitive detection.

Hepatitis D virus

HDV, also known as the *delta hepatitis virus*, is a defective 1.7-kb ssRNA virus that requires HBV for replication. HDV requires the HBV HBsAg for its envelope. HDV is the sole member of the genus *Deltavirus*, and the genus is not currently assigned to a family. HDV is transmitted primarily via parenteral routes, although transmission through mucosal contact has been implicated in epidemics in endemic areas. At-risk groups in the United States are primarily IV drug users, although limited numbers of MSM in certain parts of the country are also at risk. Because of overlap in the clinical presentation, presumed low incidence of infection, and lack of an effective surveillance mechanism, current epidemiologic data on HDV are minimal.

HDV infection is rare but usually severe and results in acute disease, with a fatality rate of 5%, or chronic symptoms progressing to cirrhosis in two thirds of those infected. The infection can occur in one of two clinical forms: co-infection or superinfection. In co-infection, the patient is simultaneously infected by HBV and HDV. In superinfection, HDV infection develops in a patient with chronic HBV infection. Patients with superinfection have a more severe acute infection and have a higher risk of fulminant hepatitis compared with patients with co-infection. Long-term carriers of HBV who become superinfected with HDV also develop chronic HDV infection, which increases their chance of developing cirrhosis.

Diagnosis of HDV infection requires serologic testing for specific HDV antibody. Commercial tests are available for IgG anti-HDV antibody. Reference laboratories may offer IgM anti-HDV antibody testing and PCR assay for HDV. Table 29.9 presents the interpretation of HDV serologic

Table 29.8 Interpretation of hepatitis B virus serologic markers

HBsAg	HBeAg	Anti-HBc	Anti-HBc IgM	Anti-HBs	Anti-HBe	Interpretation
–	NA	–	–	–	NA	No previous infection with HBV or early incubation
–	NA	+	–	±	NA	Convalescent or past infection
–	NA	–	–	±	NA	Immunization to HBsAg
+	–	–	±	–	–	Acute infection
+	+	±	+	–	–	Acute infection, high infectivity
+	–	±	+	–	+	Acute infection, low infectivity
+	+	+	–	–	–	Chronic infection, high infectivity
+	–	+	–	–	+	Chronic infection, low infectivity

–, Negative; +, positive; ±, positive or negative; *anti-HBc*, antibody to hepatitis B core antigen; *anti-HBe*, antibodies against hepatitis B envelope antigen; *anti-HBs*, antibody to hepatitis B surface antigen; *HBeAg*, hepatitis B envelope antigen; *HBsAg*, hepatitis B surface antigen; *IgM*, immunoglobulin M; *NA*, not applicable.

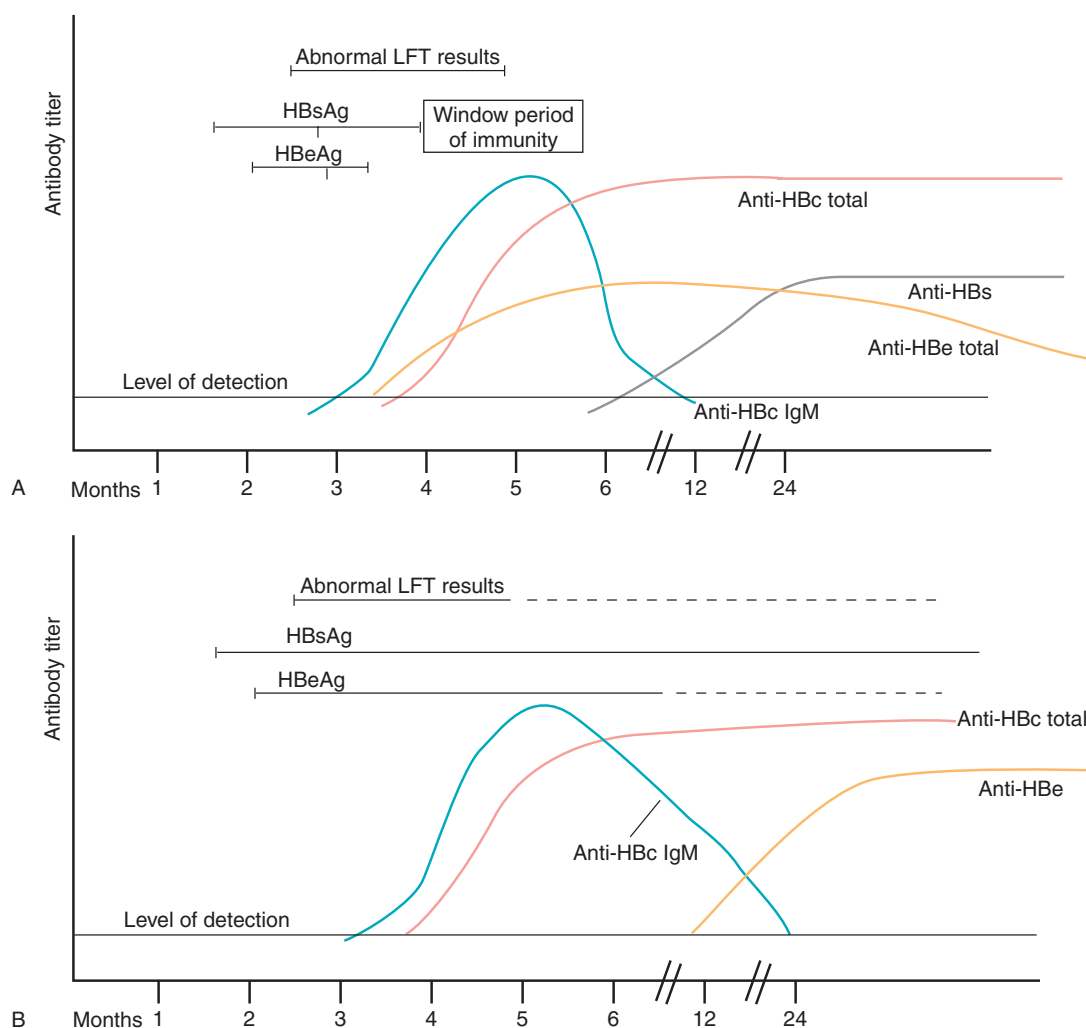


Fig. 29.26 Serologic evaluation of hepatitis B virus infection showing the increase and decrease in levels of detectable antibodies. **A**, Serologic presentation in acute hepatitis infection with resolution. **B**, Serologic presentation in chronic hepatitis infection with late seroconversion. *Anti-HBc*, Antibodies against hepatitis B core antigen; *anti-HBe*, antibodies against hepatitis B envelope antigen; *anti-HBs*, antibodies against hepatitis B surface antigen; *HBeAg*, hepatitis B envelope antigen; *HBsAg*, hepatitis B surface antigen; *LFT*, liver function test.

markers; Fig. 29.27 depicts serologic presentations of HDV co-infection and superinfection. Detection of HDV RNA is done with RT-PCR, and diagnosis using this method is becoming more common from liver tissue.

Hepatitis C virus

After methods for diagnosing HAV and HBV became available, it was apparent that these two viruses were not

responsible for all hepatitis cases, especially those related to blood transfusions. The resulting disease was termed *non-A, non-B (NANB) hepatitis*. The diagnosis of NANB hepatitis was primarily one of exclusion. In 1974, without any direct evidence, scientists predicted that a type C hepatitis virus must exist. Then, 15 years later, with the aid of molecular and cloning techniques, the genomic sequence of HCV was determined before the virus was ever seen with an electron microscope.

HCV is an ssRNA virus in the genus *Hepacivirus*, family *Flaviviridae*; it accounts for about 90% of all previous cases of NANB hepatitis. There has been a gradual increase in the annual incidence of acute HCV infections in the United States since 2012. In 2019, 4136 cases were reported, but the CDC estimates that 57,500 acute cases occurred. Worldwide, as many as 1.5 million new cases may develop each year, and an estimated 58 million people have chronic HCV. Although perinatal and sexual transmission of infection occur and parenteral transmission has been identified as a major route for infection, HCV antibodies have been detected in patients in

Clinical variant	Serologic markers			
	Anti-HBc IgM	HBsAg	Anti-HDV	Anti-HDV IgM
Co-infection	+	+	+	+
Superinfection	–	+	+	NA

Anti-HBc, antibody to hepatitis B core antigen; *HBsAg*, hepatitis B surface antigen; *HDV*, hepatitis D virus; *IgM*, immunoglobulin M; *NA*, not applicable.

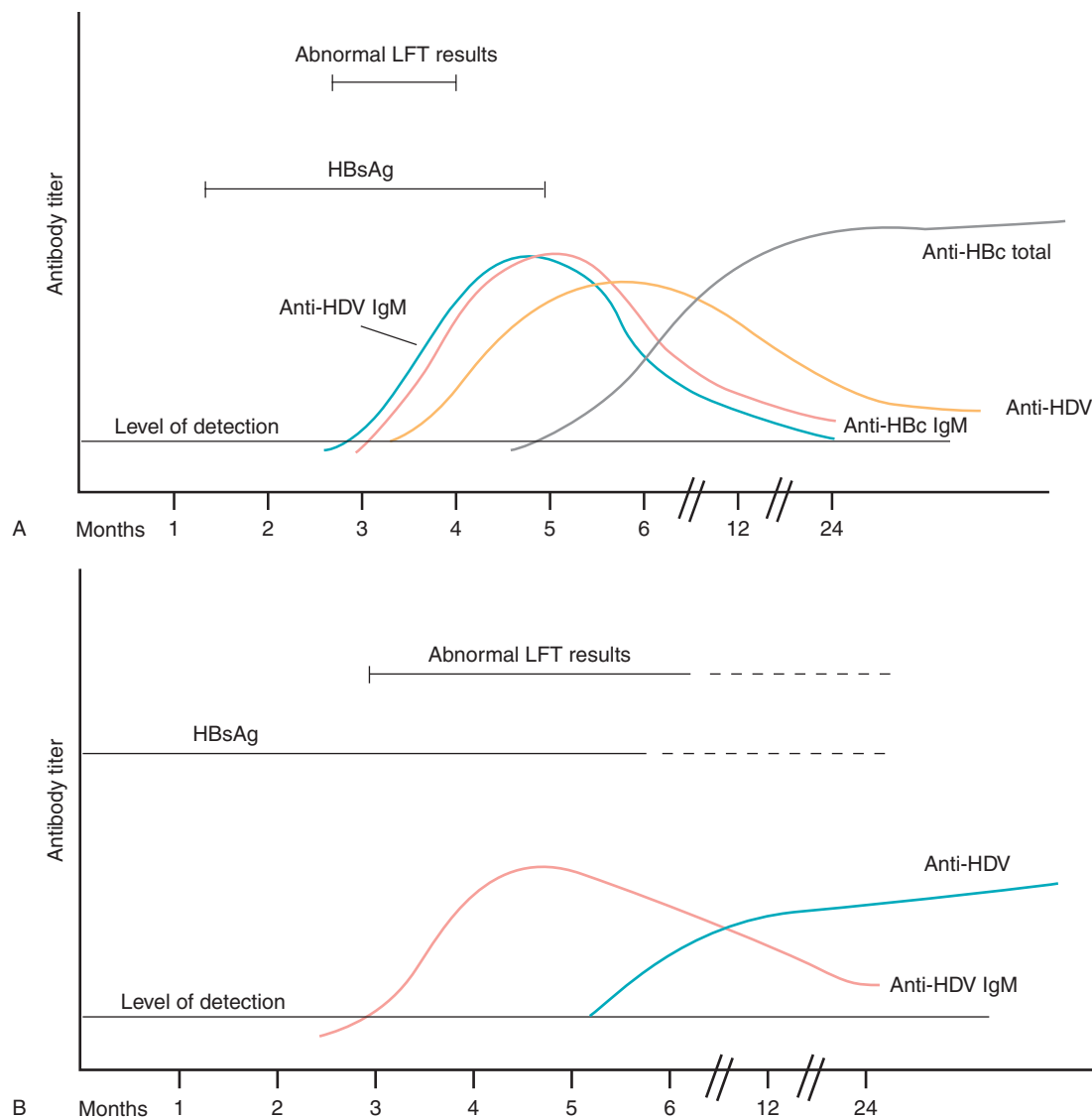


Fig. 29.27 Serologic evaluation of hepatitis D virus (HDV) infection showing the persistence of detectable antibodies. **A**, Hepatitis B virus (HBV)-HDV co-infection. **B**, HBV-HDV superinfection. *Anti-HBc*, Antibodies against hepatitis B core antigen; *HBsAg*, hepatitis B surface antigen; *IgM*, immunoglobulin M; *LFT*, liver function test.

whom the routes of transmission are poorly understood or who have no evidence of identifiable risk factors.

Symptoms can be subtle and may take time to become apparent. About 50% of HCV-positive patients become long-term carriers, and about 20% to 30% of patients with chronic infections develop cirrhosis. Cirrhosis is a major risk factor for hepatocellular carcinoma. In 2019, over 100,000 new chronic HCV infections were recorded in the United States. It is estimated that the number of U.S. citizens living with chronic HCV is 2.4 million, but it might be as many as 4.7 million. Among the three hepatitis viruses—HAV, HBV, and HCV—HCV has the highest mortality rate.

Gene amplification tests prove that HCV RNA appears in newly infected patients in as little as 2 weeks. However, most infections are detected by serologic testing. HCV is less immunogenic than HBV. Antibodies to HCV appear in about 6 weeks in 80% of patients and within 12 weeks in 90% of patients. HCV infection does not produce persistent, life-long levels of antibody; rather, persistence of anti-HCV antibody is linked to the presence of replicating HCV.

EIAs that detect serum antibodies to HCV proteins are available as screening tests; however, they can have a low sensitivity. Second-generation immunoblot assays use recombinant and/or synthetic proteins to detect anti-HCV antibodies. Currently, third-generation tests are more sensitive and accurate and are commonly used. These assays also employ recombinant proteins but have an improved version of NS3 and have added NS5. Confirmation currently relies on nucleic acid amplification testing. Figure 29.28 depicts the immunologic profile of HCV infection.

Patients with chronic HCV infection were previously treated with interferon, with or without ribavirin. First approved in 2011, direct-acting antiviral (DAA) therapy has proven highly effective in curing HCV infection. DAAs are molecules that target specific nonstructural proteins of the virus, which results in disruption of viral replication and infection. There are four classes of DAAs defined by their mechanism of action and therapeutic targets.

Hepatitis E virus

HEV is a small (32 to 34 nm), naked, ssRNA virus classified in the genus *Hepevirus*, family *Hepeviridae*. HEV is transmitted via the fecal-oral route, particularly through contaminated drinking water. HEV has been identified as the cause of epidemics of enterically transmitted hepatitis in developing countries in Asia, Africa, and Central America. Although the virus has not been associated with outbreaks in the United States, it has been linked to sporadic cases in travelers returning from endemic areas.

HEV causes an acute, self-limiting disease with clinical symptoms like those of HAV. The incubation period is 2 to 9 weeks. Signs and symptoms of HEV infection include fever, malaise, nausea, vomiting, jaundice, and dark-colored urine. No specific antiviral therapy is available; patients receive supportive therapy. Viral shedding in feces has been shown to persist for several weeks. The mortality rate is 1% to 3% overall, with a higher likelihood of death in women who are pregnant (15% to 25%). Epidemics affect primarily young to middle-age adults. An ELISA test is available to detect IgG and IgM anti-HEV antibodies, although HEV testing is not currently performed in diagnostic laboratories in the United States.

Other hepatitis viruses

Evidence for HGV was derived originally from a patient with NANB hepatitis. This RNA virus, now known as GB virus type C (GBV-C), is a member of the family *Flaviviridae*. GBV-C viremia has been demonstrated worldwide in 0.6% to 14% of blood donors, depending on the geographic location. However, infection does not seem to be common in the United States. The clinical significance of the virus is still under investigation. Experimental RT-PCR tests can be performed to detect the virus, but routine testing is not yet recommended.

The most recently identified hepatitis viruses are SEN virus and TTV. SEN virus has a circular DNA genome. It is

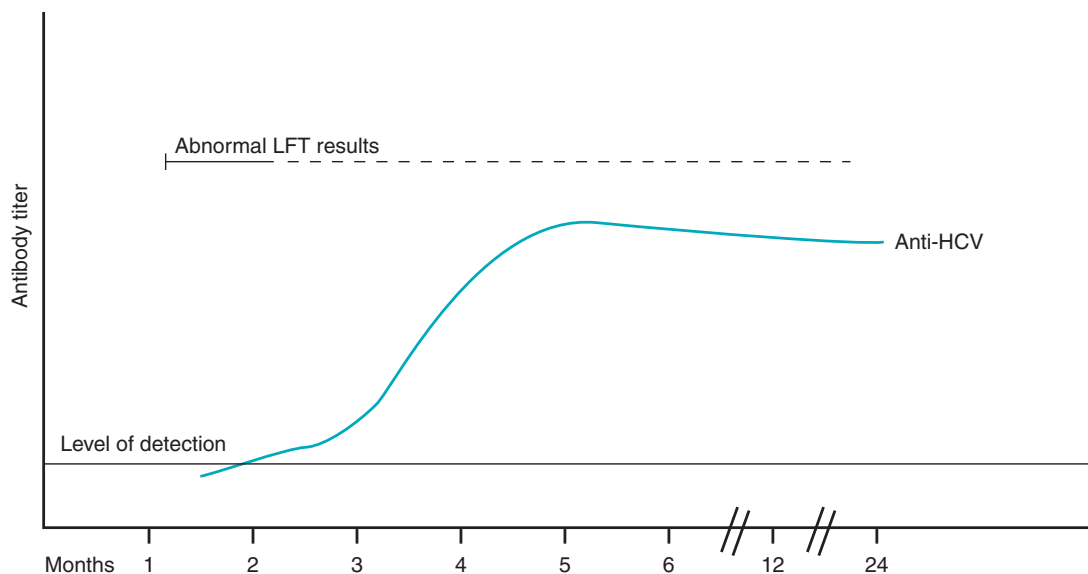


Fig. 29.28 Serologic evaluation of hepatitis C virus (HCV) infection showing the persistence of detectable antibodies, indicating the presence of replicating HCV. LFT, Liver function test.

bloodborne, and although originally suspected to be a cause of hepatitis, it has not been definitively linked to any human disease. Transmission appears to be linked to blood transfusion. About 30% of patients with HIV infection have antibodies to the SEN virus. TTV was first identified in the serum of a Japanese patient in 1997. It is an ssDNA virus related to animal circoviruses. The role of TTV in human disease is unknown but may be associated with some cases of post-transfusion hepatitis.

Prions

Prions are not viruses but are proteinaceous infectious particles that cause a group of diseases in mammals called *transmissible spongiform encephalopathies* (TSEs). The name *prion* (from the words **protein** and **infection**) was coined by Stanley B. Prusiner in 1982. Examples of TSEs include kuru and Creutzfeldt-Jakob disease (CJD) in humans, scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle, and chronic wasting disease in deer and elk. BSE, also referred to as *mad cow disease*, was responsible for the loss of hundreds of thousands of cattle in the United Kingdom, most notably during the 1980s.

An increase in a variant form of CJD (vCJD) in the United Kingdom was noted at about the same time as the surge of BSE. It appears that the agent of scrapie crossed species and infected cows and that it is the causative agent in BSE-infected humans, causing vCJD. Between 1993 and 2018, 26 cases of BSE were discovered in North America: 6 cases in the United States and 20 in Canada. TSEs are known to be acquired via ingestion, although some prions may also enter the body by other routes. Sheep offal used in cattle feed was the likely cause of the prion moving from sheep to cattle.

TSEs are characterized by progressive, relentless degeneration of the CNS, which is ultimately fatal. Typical histopathology is neuronal vacuolation and eventual loss of neurons, accompanied by proliferation and hypertrophy of fibrous astrocytes. The prion protein (PrP) found in animals with TSE is often referred to as PrP^{Sc}, named after the prion found in sheep with scrapie. Animals, including humans, have a similar but normal protein found on cells of the CNS, referred to as PrP^C. Ingested PrP^{Sc} is absorbed into the bloodstream and makes its way into the CNS. PrP^{Sc} converts PrP^C into PrP^{Sc}, which is released by neuronal cells. PrP^{Sc} accumulates in the CNS, producing amyloid plaques and the characteristic histopathology.

The diagnosis of TSE is often based on clinical findings. Routine analysis of CSF is nonrevealing. Many patients with CJD will have 14-3-3 proteins in CSF; however, these proteins are not specific for CJD. The presence of these proteins in CSF is a marker for rapid neuronal cell death, present not only in CJD but also in encephalitis and conditions with CNS hemorrhaging. Histopathology staining and PrP^{Sc} immunostaining remain the most specific diagnostic methods. Other detection methods include antigen detection, serologic testing, and nucleic acid sequencing for inheritable forms. Recent findings have suggested the excretion of TSEs in urine.

The risk of infection to laboratory personnel is low, but material suspected of containing these agents must be handled carefully. Prions are extremely resistant to inactivation; even 2 hours in a steam autoclave might not inactivate all

prions in a specimen. Exposure to household bleach with more than 20,000 parts per million available chlorine or 1 M sodium hydroxide is recommended. It is not recommended to test any specimens from a suspected case of prion disease until it is ruled out because instrumentation cannot be sufficiently decontaminated, and it presents a risk of infection to staff.

Treatment and management of viral infections

Viruses have evolved to replicate inside host cells. Since the virus uses host machinery to replicate, most drugs targeting viruses have the potential to affect the host cell as well. Viruses with very high mortality rates (e.g., Ebola virus) are often eradicated equally rapidly as the survival of virus relies on effective transmission to new hosts. Therefore most pathogenic viruses eventually mutate to milder infections and become more contagious. This is the trend being observed in the ongoing COVID-19 pandemic. Most mild viral illnesses are not treated; instead, supportive care is provided for the viral cycle to be completed, and quarantine of infected individuals is recommended to break the transmission cycle.

Antiviral therapy

Some viral infections are treatable, especially if the laboratory can rapidly identify the pathogen. Antiviral compounds must target an essential viral replicative mechanism without destroying or damaging uninfected host cells. Several antiviral agents resemble nucleosides used in viral replication. The viruses insert these nucleoside analogues into their own nucleic acid, resulting in disruption of viral replication. Other antivirals are non-NRTIs, which also disrupt viral replication. Phosphonoformic acid (foscarnet) is an analogue of pyrophosphate that acts directly as a DNA polymerase inhibitor. Other antiviral compounds inhibit viral replication by targeting key viral proteins (e.g., protease inhibitors). Some commonly used antiviral agents are given in [Table 29.10](#). A number of antiviral drugs have been developed, and several are in clinical trials for the treatment of COVID-19.

Just as antibacterial agent use increases the risk of drug resistance in bacteria, the use of antiviral agents can result in viruses that become resistant to therapy. As more antiviral agents become available, antiviral susceptibility testing will become increasingly important. For example, foscarnet is currently being used to treat infections caused by HSV strains resistant to acyclovir and to treat CMV strains resistant to ganciclovir.

Vaccines

Development of vaccines for highly contagious viruses is a top priority in the management of clinically relevant viral infections. A vaccine is composed of a highly weakened attenuated virus or virus component that can trigger an immune response. When the body encounters the actual infection, the host will mount a heightened (anamnestic) immune response, ultimately leading to clearance of the infectious agent. It is important to note that vaccines prevent disease

Table 29.10 Examples of antiviral compounds

Antiviral	Inhibits	Active against
Acyclovir	DNA polymerase	HSV, VZV
Cidofovir	DNA polymerase	CMV (retinitis)
Famciclovir	DNA polymerase	HSV-2
Ganciclovir	DNA polymerase	CMV (retinitis)
Valacyclovir	DNA polymerase	HSV-2
Idoxuridine, trifluridine	DNA synthesis (DNA base analogue)	HSV (keratitis)
Amantadine, rimantadine	Uncoating	Influenza A (treatment and prophylaxis)
Interferon- α	Viral replication (multiple mechanisms)	HPV (genital warts); chronic HCV, Kaposi sarcoma
Ribavirin	Viral replication (multiple mechanisms)	RSV, CCHF
AZT or ZDV	Reverse transcriptase	HIV
ddI	Reverse transcriptase	HIV
ABC	Reverse transcriptase	HIV
3TC	Reverse transcriptase	HIV
d4T	Reverse transcriptase	HIV
ddC	Reverse transcriptase	HIV
FTC	Reverse transcriptase	HIV
TDF	Reverse transcriptase	HIV
Indinavir	Proteases	HIV
Nelfinavir, ritonavir	Proteases	HIV, CoV-2
Saquinavir	Proteases	HIV
Lamivudine		Chronic HBV
Adefovir		Chronic HBV
Remdesivir	Adenosine analog	CoV-2

ABC, Abacavir sulfate; AZT, azidothymidine; d4T, stavudine; CCHF, Crimean-Congo hemorrhagic fever; CMV, cytomegalovirus; CoV-2, coronavirus 2; ddC, zalcitabine; ddI, didanosine; FTC, emtricitabine; HCV, hepatitis C virus; HIV, human immunodeficiency virus; HPV, human papillomavirus; HSV, herpes simplex virus; RSV, respiratory syncytial virus; 3TC, lamivudine; TDF, tenofovir; VZV, varicella-zoster virus; ZDV, zidovudine.

successful vaccines like smallpox vaccine and polio vaccine were tremendously successful, leading to eradication or near eradication of these viral diseases worldwide. Most recently, three COVID-19 vaccines were developed, two of which (Moderna [Cambridge, MA] and Pfizer [New York, NY]-BioNT [Germany]) used mRNA coding for a surface protein of SARS-CoV-2. The third, from Johnson and Johnson (New Brunswick, NJ), used an attenuated virus. These vaccines have been instrumental in curbing the spread and mortality of COVID-19. Global efforts to invest in research and development of effective vaccines as well as increasing awareness to the public about the safety and efficacy of vaccinations is a key step to prevent future pandemics.

POINTS TO REMEMBER

- Viruses have been responsible for several pandemics including influenza viruses and coronaviruses.
- Clinical virology services can consist of rapid antigen detection, NAATs, antibody detection, or cell cultures.
- Clinically significant viruses can be isolated from patients with signs and symptoms commonly thought to be associated with bacterial infections, including pneumonia, GI disorders, cutaneous lesions, sexually transmitted infections, and sepsis.
- Members of the family Herpesviridae produce life-long, latent infections.
- Most cases of cervical cancer are linked to HPV, the causative agent of genital warts.
- Some viruses mutate rapidly, resulting in new strains, which can be challenging to contain or treat.
- Retroviruses (e.g., HIV) replicate with reverse transcriptase, which uses viral RNA as a template to make a complementary DNA strand.
- Arboviruses are those viruses transmitted by the bite of arthropods, such as mosquitoes.
- Many emerging infections are caused by zoonotic viral agents that are unexpectedly transplanted into a susceptible human population.
- The hepatitis viruses are a diverse collection of viruses grouped together because they all infect primarily the liver. Laboratory diagnosis is based on serologic markers.
- Antiviral compounds can treat numerous viral infections, but resistance has been seen.
- Vaccines against viruses is an effective way to manage viral infections and severity.

LEARNING ASSESSMENT QUESTIONS

1. Which of the following serologic markers should be positive following administration of the HBV vaccine?
 - a. HBeAg
 - b. HBcAg
 - c. Anti-HBs
 - d. Anti-HBc
2. Which of the following conditions is most often associated with rotavirus?
 - a. Infant diarrhea
 - b. Paralysis in children

but not infection. Those infected after vaccination are usually asymptomatic, or have a milder form of disease, and eliminate the infectious agent quickly. They are much less likely to transmit the agent to other hosts. Once a significant percent of the population develops immunity, either by vaccination or surviving the disease, the infection rate becomes very low. This concept is referred to as *herd immunity*.

Unfortunately, due to the high rate of mutations, effective vaccines cannot be developed for all viruses. A number of

- c. Infant respiratory infections
- d. Infectious mononucleosis-like symptoms in young adults
3. Which virus causes fifth disease?
 - a. Molluscum contagiosum
 - b. Parvovirus B19
 - c. Measles virus
 - d. Varicella-zoster virus
4. During the “core window” of acute hepatitis B virus infection, what is the primary marker for diagnosis?
 - a. HBsAg
 - b. HBeAg
 - c. Anti-HBs
 - d. Anti-HBc
5. Reactivation of the virus causing chickenpox results in which condition?
 - a. Shingles
 - b. Infectious mononucleosis
 - c. Dengue fever
 - d. Polio
6. The presence of Kaposi sarcoma in a young adult is suggestive of which illness?
 - a. West Nile fever
 - b. Ebola hemorrhagic fever
 - c. Acquired immunodeficiency syndrome
 - d. Coronavirus disease
7. A patient suspected of having hepatitis has a positive anti-hepatitis C virus (HCV) antibody test. Which of the following should be done next?
 - a. Report the patient as HCV positive.
 - b. Perform an anti-HBs test to rule out a false positive
 - c. Request a second blood sample to repeat the test.
 - d. Confirm the result with a nucleic acid amplification test for HCV.
8. What is the greatest risk for developing dengue hemorrhagic fever?
 - a. History of dengue fever with a different serotype
 - b. Older than 50 years of age
 - c. Diabetes
 - d. Obesity
9. What is the most common complication of West Nile fever?
 - a. Pneumonia
 - b. Neuroinvasive disease
 - c. Chronic hepatitis
 - d. Osteomyelitis
10. Which of the following specimens is best to recover cytomegalovirus?
 - a. Nasopharyngeal swabs
 - b. Bronchial wash
 - c. Buffy coat
 - d. Saliva
11. Which opportunistic infections or conditions are used as indicators of acquired immunodeficiency syndrome (AIDS)?
12. Which immunologic markers are used to diagnose human immunodeficiency virus (HIV) infection?
13. What disease does Epstein-Barr virus (EBV) produce? What complications can result from EBV infections?
14. How is acute hepatitis B virus (HBV) infection differentiated from chronic infection? Which markers indicate resolution of the infection?

15. What are the differences between classic dengue fever and dengue hemorrhagic fever?
16. What are the methods commonly used to diagnosis rabies?
17. Which types of infections are caused by human papilloma-virus (HPV)?
18. Which viruses have the potential for latency?
19. Why are vaccines for influenza not always effective?
20. What type of vaccines showed highest success against SARS-CoV-2 virus?

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Agents of bioterror and forensic microbiology

Christopher J. Woolverton and Donald C. Lehman

CHAPTER OUTLINE

Bioterrorism readiness and the clinical laboratory, 754

Laboratory response network, 756

General characteristics of biological threat agents, 757

Biological risk management, 758

Biosafety, 759

Biosafety levels, 759

Agents of bioterror, 760

Bacillus anthracis, 760

Burkholderia species, 762

Clostridium botulinum neurotoxin, 764

Ebola virus and Marburg virus, 764

Francisella tularensis, 766

Variola virus, 767

Yersinia pestis, 769

Other biological agents and toxins, 771

Pandemic preparedness and bioterrorism readiness, 771

Concluding thoughts, 772

Forensic microbiology, 772

Bibliography, 774

9. Describe the evolution of the use of biological agents throughout history.
10. Differentiate the roles of the three levels of Laboratory Response Network (LRN) laboratories: sentinel, reference, and national.
11. Describe the roles of organizations that offer support for the preparation and investigation of potential bioterror events.
12. Compare the pathogenesis and the mode of transmission of each bioterror agent.
13. Identify specific virulence factors unique to each tier 1 bioterror agent.
14. Associate the clinical manifestations of infection with the bioterror agents listed in this chapter.
15. Identify the necessary characteristic growth patterns, Gram stain morphology, and biochemical test results to rule in or rule out *Bacillus anthracis*, *Yersinia pestis*, and *Francisella tularensis*.
16. Based on a health care provider's suspicion, identify the ideal specimens to submit for testing and any special precautions or safety procedures to identify disease caused by variola virus, Ebola virus or Marburg virus, and *Clostridium botulinum* toxin.
17. Assess the impact of the anthrax bioterror incident on the modification or development of new policies and procedures dealing with bioterror threats.
18. Discuss the role of forensic microbiology.

OBJECTIVES

After reading and studying this chapter, you should be able to:

1. Identify the four levels of biosafety.
2. Describe the differences in special laboratory practices, safety equipment, and laboratory facilities.
3. Develop a biological risk assessment to recognize the adequate biosafety level (BSL) requirements for specific agents.
4. Summarize the features of an ideal bioterror agent.
5. Explain how CRISPR/CAS technology can be used to enhance the virulence of a bioterror agent.
6. Explain the differences among the categories of bioterror agents.
7. Compare the features of the four categories of BSL laboratories.
8. Compare the efficiency of the different routes used to disseminate bioterror agents.

KEY TERMS

Biocrime	Buboes
Biological agent	Bubonic plague
Biological risk assessment	Eschar
Biological warfare	Glanders
Biorisk	Inhalation anthrax
Biorisk management	Laboratory Response Network (LRN)
Biosafety	Mediastinitis
Biosafety levels (BSLs)	Melioidosis
Bioterrorism	Pneumonic plague
Biothreat	Risk group
Black Death	Select agents
Botulinum toxin	

Tier 1 agents
Tularemia
Vaccinia

Variola major
Variola minor

Case in point

On October 16, a 56-year-old African American man experienced fever, chills, headache, sore throat, and malaise. Symptoms progressed to difficulty in breathing, night sweats, nausea, and vomiting. He went to the hospital on October 19. At admission, he was afebrile, and his heart rate was 100 beats per minute. Physical examination found decreased breath sounds and rhonchi. His complete blood count was unremarkable, and serum chemistries and renal function tests were normal. Arterial blood gas did not show hypoxia. Chest radiography revealed a widened mediastinum, and computed tomography showed mediastinal edema. Specimens were collected for blood culture, and gram-positive bacilli presumptively identified as *Bacillus anthracis* grew within 11 hours. Therapy with ciprofloxacin, rifampin, and clindamycin was started. On October 21, the patient developed respiratory distress, and he was treated with diuretics and corticosteroids, and underwent thoracentesis. The patient eventually recovered.

Issues to consider

After reading the patient's case history, consider:

- Why *Bacillus anthracis* can be classified as both a BSL-2 and a BSL-3 agent
- Whether *B. anthracis* meets all features of an ideal bioterror agent
- What tests a sentinel laboratory should perform to rule out *B. anthracis*
- What clinical manifestations are indicative of a potential inhalational case of anthrax and whether a case, when identified, is always considered a bioterror event

Bioterrorism readiness and the clinical laboratory

The announcement of a severe acute respiratory syndrome (SARS)-like viral outbreak in Wuhan, China, at the end of 2019 triggered many people to wonder about its origin. The subsequent pandemic brought confusion, fear, and panic. As the sequence of events unfolded and infected individuals began to die, some wondered if the deadly virus had been intentionally released. Clinical laboratories worldwide scrambled to identify the pathogen. The initial disease pattern and epidemiology closely mirrored the plots of several apocalyptic tales of science fiction, leaving people to wonder if another bioterrorism attack had been perpetrated. The virus was named SARS-coronavirus-2 (CoV-2). Coronavirus disease 2019 (COVID-19) was labeled zoonotic; its origin is still unknown.

Bioterrorism is the intentional or threatened use of bacteria, viruses, fungi, or toxins from living organisms to produce death or disease in humans, animals, or plants. Implicit in this definition is the intention to cause civil and economic instability leading to fear, panic, and chaos. Bioterrorism can be classified as overt or covert. In overt bioterrorism, the impact will

be immediate, and there will be early recognition of the event, generally by emergency response personnel. Additionally, a group or society will often claim responsibility for such an event. Covert events pose more of a challenge. There may not be an initial announcement of an attack, early health care data may not differentiate it from a natural epidemic organism, and clinical microbiologists and health care providers will most likely be the first to suspect the attack. During covert bioterrorism, the recognition as well as the response could be delayed for days or weeks, allowing the disease to spread throughout a population.

To the terrorist, biological agents may seem easy to acquire and inexpensive to produce, and they can offer an alibi or escape as the agent multiplies in the infected person during its incubation period. To this end, a bioterror event may initially mirror a natural disease outbreak, but it would quickly overwhelm underprepared medical services. The clinical laboratory may not even have the correct reagents or media to support the correct diagnosis of the disease. We will learn later in the chapter how this has been addressed and how the clinical laboratorian can be prepared for a potential bioterrorism event.

Biological warfare is the deliberate use of bacteria, viruses, fungi, or toxins from living organisms to produce death or disease in humans, animals, or plants by a state-level organization to gain a military advantage and/or political gain. There are several incidents of biological warfare recognized throughout human history. In one of the first recorded cases, the Assyrian army of the sixth century B.C. poisoned the wells of their enemies with a rye ergot fungus. This specific fungus produced an alkaloid hallucinogen known as lysergic acid diethylamide (LSD). The hallucinogen distracted their enemies, thus granting easy victories to the Assyrians. In the fourth century B.C., Scythian army archers dipped their arrows in poisons made from blood, manure, and even decomposing bodies.

The most historically recognized biowarfare event occurred in the 14th century, when the Tartar army laid siege to the walled city of Caffa (modern-day Feodosiya, Ukraine). Unable to enter the fortified city, the army catapulted bodies of Mongol plague victims over the city wall to force the Genoese Christians out of their stronghold. History reveals that the Mongol corpses did not really transmit plague. Likely, the disease was started as rats crawled into the walled city, spreading the disease via bacteria-infected flea vectors (Fig. 30.1). The surviving Genoese, indeed, fled the city, but by way of the seaport side of the city, where ships carried them into the Black Sea. Unfortunately, it is believed that the rats and their infected fleas stowed aboard the ships, traveled with the Genoese to other European ports, and thus aided in the spread of the **Black Death** throughout Europe.

History also remembers the notorious misuse of microorganisms to control military outcomes during the French and Indian War (in the United States), when Sir Jeffery Amherst, the commander of British troops in North America, wrote a letter to Colonel Henry Bouquet suggesting the use of smallpox-contaminated materials to kill Native Americans sympathetic to the French. In 1763, Captain Simon Ecuyer, stationed at Fort Pitt, Pennsylvania, collected the blankets and a handkerchief used by a soldier at Fort Pitt who had recently died of smallpox. These contaminated items were presented to the Native American Delaware tribe, causing an outbreak and



Fig. 30.1 Scanning electron micrograph of a flea vector, which may transmit *Yersinia pestis* to humans via a blood meal ($\times 20,000$). (Courtesy Janice Haney Carr and Centers for Disease Control and Prevention, Atlanta, GA.)

nearly decimating the tribe so the British could claim a military victory against a French military post.

The “modern era” of biological weapons began during World War I, when Germany was believed to have used *Vibrio cholerae* and *Yersinia pestis* against humans and *B. anthracis* and *Burkholderia mallei* against animals. *B. mallei* was used to infect the horses and mules of Allied countries, thus preventing them from transporting supplies and equipment to the European war front. Despite a 1925 Geneva Protocol signed by 108 nations prohibiting the use of biological agents during war, several countries began biological warfare research programs in the 1920s that continued through World War II (1939 to 1945) and the Korean War (1950 to 1953). It was not until 1972 when a new document, summarizing the efforts of many nations and meant to supplement the 1925 Geneva Protocol, was presented for international ratification. Known as the Biological Weapons Convention, it set as its goal the banning of all offensive biological weapons. By 1975, 22 countries had ratified its *general-purpose criteria* that no signer of the agreement would develop, produce, stockpile, or otherwise acquire or retain (1) microbial or other biological agents or toxins that have no peaceful purpose and (2) weapons, equipment, or means of delivery designed to use such agents of toxins for hostile purposes or in armed conflict. With the hope that the Biological Weapons Convention would prevent state-sponsored biowarfare, the world continued to worry as isolated acts of **biocrime** (i.e., acts of bioterrorism committed by individuals and small groups) became more frequent in the late 20th century.

In 1984, the followers of Bhagwan Shree Rajneesh committed biocrimes when they used *Salmonella enterica* serovar Typhimurium as a weapon to help influence election results

in the Dalles, Oregon, community. By their covertly spraying liquid *Salmonella* cultures on restaurant salad bars, 751 persons developed culture-positive salmonellosis. Their plan to influence the election ultimately failed, but they raised serious concerns about how food and water could be used to deliver biological agents to unsuspecting victims.

Internationally, other incidents raised growing concerns that a bioweapons deployment program would succeed. The Japanese cult Aum Shinrikyo successfully deployed sarin gas in the subways of Tokyo in 1995, killing 12 and injuring almost 5000 people. The Aum also obtained and experimented with **botulinum toxin**, *B. anthracis*, and *Coxiella burnetii* and attempted to obtain Ebola virus (EBOV) from Zaire (now the Democratic Republic of the Congo). From 1990 to 1995, they tried several times to deploy botulinum toxin and anthrax spores in cities, all unsuccessfully.

One biocrime still remembered by many is the 2001 attacks on politicians and media celebrities with spores of *B. anthracis* sent through the U.S. mail. Envelopes laced with spores resulted in 22 cases of anthrax in five cities on the eastern seaboard, and dozens of buildings were contaminated. The U.S. Congress was temporarily closed, and the U.S. Postal Service was disrupted for years. Ultimately, five people died because of this attack and \$6 billion was spent in response costs. These and other events led the U.S. Department of Health and Human Services (DHHS) to enhance the **Laboratory Response Network (LRN)**, a national detection and mitigation program that could rapidly respond in the event of a bioterrorism crisis ([Box 30.1](#)).

Case check 30.1

Throughout the years, biological agents have been used for many purposes, such as to cause public panic, incapacitate individuals, and provide an advantage over one's enemy during military operations. The anthrax letters mailed in 2001 caused a great amount of panic and fear among the public. Many hospitals and laboratories were overwhelmed by individuals concerned that they had been exposed (often categorized as the “worried well”). The Case in Point is an example of the criminal use of *B. anthracis*.

BOX 30.1 Key indicators of a potential bioterror or biocrime event

- A disease entity that is unusual or that does not occur naturally in a given geographic area or combinations of unusual disease entities in the same patient populations
- Multiple disease entities in the same patients, indication that mixed agents might have been used in the event
- Above-normal rates of morbidity and mortality relative to the number of personnel at risk or within a population that inhabits the same area
- Data suggesting a massive point source outbreak
- Apparent aerosol route of infection
- Illness limited to localized or circumscribed geographic areas with filtered air supplies or closed ventilation systems
- Sentinel dead animals of multiple species
- Absence of a competent natural vector in the area of the outbreak (for a biological agent that is vector-borne in nature)

From U.S. Army Medical Research Institute for Infectious Diseases. (2000). *Biological warfare and terrorism medical issues and response satellite broadcast*. Ft. Detrick, MD: USAMRIID.

Laboratory response network

In an effort to increase responsiveness, standardize reagents and protocols, and create the ability nationwide to rapidly detect the deployment of biological weapons in the United States and its territories, infectious disease experts in academia, national public health experts, DHHS agency representatives, civilian and military intelligence experts, and law enforcement officials met in the late 1990s, in response to Presidential Decision Directive 39, to discuss the threat potential of infectious agents to civilian populations. One result was the reassessment of biological agents for their ability to cause harm in individuals and communities, should they be used as agents of bioterrorism. By law, the biological agents were placed into one of three categories—A, B, or C—for initial public health preparedness efforts. Revised legislation now categorizes select agents as *tier 1* or *non-tier 1* based on several criteria, including the degree of public health threat.

A second result of those meetings was the creation of the LRN in 1999, formed by the Centers for Disease Control and Prevention (CDC), the Association of Public Health Laboratories (APHL), the Federal Bureau of Investigation (FBI), and the U.S. Army. The stated goal of the LRN was to decentralize testing capabilities and link clinical laboratories and public health laboratories to advanced-capacity clinical, military, veterinary, agricultural, water, and food testing abilities. This integrated network of laboratories can respond rapidly to bioterrorism, emerging infectious diseases, chemical terrorism, or other public health emergencies. The LRN is a tiered response network (Fig. 30.2).

Thousands of laboratories in the United States perform diagnostic testing. Most modern hospital laboratories serve

in this “sentinel” capacity. By virtue of its daily contact with patient specimens, effective diagnostic testing, good laboratory practice, and clinical problem-solving skills, the local clinical (sentinel) laboratory is likely the first point of detection should victims of bioterrorism seek infectious disease diagnostics. Here, detection of locally atypical infectious organisms will initiate specific protocols to “recognize, rule out, and refer” specific microorganisms that might be used in bioterrorism acts. The American Society for Microbiology (ASM) publishes guidelines instructing sentinel laboratories how to conduct preliminary testing to recognize and rule out these “select agents” only when they are suspected from clinical diagnoses or initial test results.

When the select agent cannot be ruled out using the sentinel laboratory protocol, then the sentinel laboratory must cease identification procedures and send the isolate to the designated LRN reference laboratory for confirmatory identification. The sentinel laboratory completes a chain of custody form and prepares specimens for shipping. The CDC and the ASM also provide guidance on the proper procedures for packing and shipping isolates for transfer to an LRN reference laboratory. Importantly, sentinel clinical laboratories do not require registration with the Select Agent Program to conduct both tier 1 and non-tier 1 select agent *diagnostic* testing if the laboratory destroys any residual specimen and destroys or transfers the confirmed select agent within 7 days of identification. Reporting of all identified select agents is still required.

The LRN reference laboratory tier contains mostly state health department laboratories that have specific biosafety equipment, standardized reagents and controls, agent-specific protocols for the identification of **biothreat** agents, and highly trained staff. These reference laboratories can perform gene amplification and antigen detection. When practical, they also rely heavily on culture identification and characterization. Currently, the LRN has over 120 domestic laboratories, representing all 50 states, and several international laboratories.

The uppermost LRN tier represents the national centers that offer the greatest resources and responses to a biological terror event. They function in the development of new diagnostic tests, the design and testing of standardized protocols, and the definitive characterization of agents unclassified at lower levels. These laboratories are at the CDC, the U.S. Army Medical Research Institute of Infectious Diseases, the Naval Medical Research Center, and approximately a dozen strategically located U.S. facilities that have high-containment laboratories to handle the most dangerous agents (Fig. 30.3).

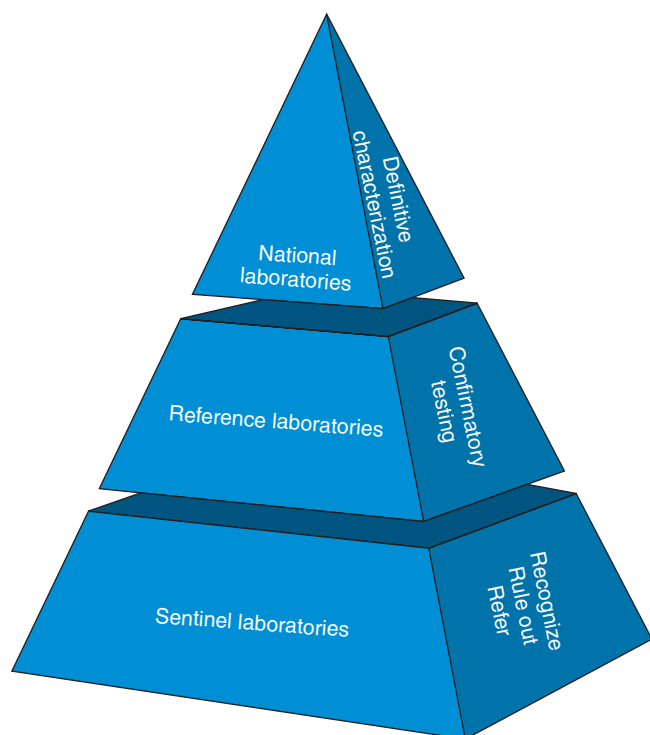


Fig. 30.2 Structure of the Laboratory Response Network.

Case check 30.2

The Case in Point demonstrates the quick and efficient reaction of sentinel facilities and member reference laboratories to organize and assess patients, samples, and tests in response to any potential threats. The laboratory that isolated *B. anthracis* in the Case in Point would be considered an LRN sentinel laboratory, which appropriately contacted the state public health laboratory (LRN reference laboratory) and referred the potential organism for confirmatory testing.



Fig. 30.3 A Centers for Disease Control and Prevention microbiologist, outfitted in an orange, airtight, self-contained, positively pressurized suit, counting viral plaques while working inside a biosafety level 4 laboratory. (Courtesy Dr. Scott Smith and Centers for Disease Control and Prevention, Atlanta, GA.)

General characteristics of biological threat agents

According to the *Code of Federal Regulations*, a **biological agent** is, by definition, any microorganism (including but not limited to bacteria, viruses, fungi, rickettsiae, or protozoa) or infectious substance, or any naturally occurring, bioengineered, or synthesized component of any such microorganism or infectious substance, capable of causing death, disease, or other biological malfunction in a human, animal, plant, or another living organism; deterioration of food, water, equipment, supplies, or material of any kind; or deleterious alteration of the environment. Some of these are recognized now as **select agents**, which are biological agents and toxins deemed by the DHHS to have the potential to pose a serious threat to public health and safety, and overlap select agents that have the potential to pose a severe threat to public health and safety, animal health, or animal products (Table 30.1). The Federal Select Agent Program oversees possession of any of these biological agents and toxins, requiring the registration of facilities that possess, use, or transfer them.

What makes biological agents so appealing to terrorist organizations? They are much less expensive to create than conventional weapons, and cultivating them may not require extensive training, expertise, or sophisticated equipment and supplies. The ideal biological agent would have high attack and fatality rates and have a very short interval between the onset of illness and death, making an accurate

and timely diagnosis very difficult. There would be limited civilian immunity and no available antimicrobial therapy, and it would be highly communicable. Finally, such an organism would be easy to produce and disseminate. Fortunately, of the known biological agents, none meet all these criteria. Thus, sooner or later, terrorist organizations may work toward devising novel weapons by using techniques like the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated proteins (Cas) gene editing or synthetic biology technology to enhance or combine the characteristics of multiple organisms or toxins.

The most efficient biological agents are transmitted by aerosols. They are more contagious than those dispersed by other methods and have the capacity to spread over a much larger targeted area. Under optimal conditions, aerosolized dispersion can affect large numbers of individuals, animals, or crops. For example, in the 1960s, the U.S. Army released *Bacillus atropeus* (formerly *B. globigii*), a nonpathogenic surrogate of *B. anthracis*, into the New York City subway system. Calculations performed subsequently indicated that over 10,000 people would have died if *B. anthracis* had been used. If such an experiment were repeated today, the number of fatalities would be much higher because of the larger population.

A main factor that determines the effectiveness of agents aerosolized outdoors is the weather. Wind currents can carry the infectious particles far from the target site, or sudden rainfall could cause the agent to fall to the ground. Agents could also be inactivated by extreme temperatures and ultraviolet (UV) light from the sun. Because bacterial spores, such as those of *B. anthracis*, are resistant to environmental stress (e.g., extreme temperatures, UV light, drying), spores are particularly attractive as bioterror agents. There is difficulty in producing even-sized particles that would be small enough to enter the alveolar air sacs and cause infection. The ideal size would be 1 to 5 μm . Particles larger than 5 μm clump together and are filtered by the upper respiratory tract or settle to the ground more quickly. Particles smaller than 1 μm are just exhaled. Thus the ideal person-to-person spread, such as with smallpox virus and **pneumonic plague** bacteria, would result from uniformly sized, 2- to 3- μm agents prepared for aerosolization.

The detection of a possible bioterror event therefore requires air, food, and water surveillance programs, laboratory scientists trained to recognize potential bioterror agents, and communication with clinicians, emergency response personnel, and infectious disease specialists. The LRN is an important

Case check 30.3

BSL-2 is recommended for activities using clinical materials and diagnostic quantities of infectious cultures. Occasionally, a potential bioterror agent, such as *B. anthracis*, may be isolated from a patient specimen under these circumstances. Because these are actively growing cultures, there is no need for the organism to sporulate. By using standard microbiological procedures, such organisms can safely be ruled in or out and referred to a public health laboratory for additional testing, if warranted. BSL-3 laboratory conditions are required to protect laboratory staff when there is a chance of aerosolization, especially when suspicious powders are tested. Environmental samples should be tested only by certified public health laboratories. The Case in Point demonstrates that the BSL-2 clinical laboratory is adequate for the initial clinical detection of most potential bioterror agents.

Table 30.1 Select agents and toxins list

U.S. Department of Health and Human Services (DHHS) select agents and toxins	Overlap select agents and toxins
Abrin <i>Bacillus cereus</i> biovar <i>anthracis</i> Botulinum neurotoxins ^a Botulinum neurotoxin-producing species of <i>Clostridium</i> ^a Conotoxins (short, paralytic α -conotoxins containing the following amino acid sequence: X ₁ CCX ₂ PACGX ₃ X ₄ X ₅ X ₆ CX ₇) <i>Coxiella burnetii</i> Crimean-Congo hemorrhagic fever virus Diacetoxyscirpenol Eastern equine encephalitis virus Ebola virus ^a <i>Francisella tularensis</i> ^a Lassa fever virus Lujo virus Marburg virus ^a Monkeypox virus Reconstructed replication-competent forms of the 1918 pandemic influenza virus containing any portion of the coding regions of all eight gene segments (reconstructed 1918 influenza virus) Ricin <i>Rickettsia prowazekii</i> Severe acute respiratory syndrome-associated coronavirus Saxitoxin South American hemorrhagic fever viruses: Chapare Guanarito Junín Machupo Sabiáa Staphylococcal enterotoxin A, B, C, D, and E subtypes T-2 toxin Tetradotoxin Tickborne encephalitis complex (flavi) viruses: Far Eastern subtype Siberian subtype Kyasanur Forest disease virus Omsk hemorrhagic fever virus Variola major virus (smallpox virus) ^a Variola minor virus (alastrim) ^a <i>Yersinia pestis</i> ^a	<i>Bacillus anthracis</i> ^a <i>Bacillus anthracis</i> Pasteur strain <i>Brucella abortus</i> <i>Brucella melitensis</i> <i>Brucella suis</i> <i>Burkholderia mallei</i> ^a <i>Burkholderia pseudomallei</i> ^a Hendra virus Nipah virus Rift Valley fever virus Venezuelan equine encephalitis virus
	U.S. Department of Agriculture select agents and toxins
	African horse sickness virus African swine fever virus Avian influenza virus Classic swine fever virus Foot-and-mouth disease virus ^a Goat pox virus Lumpy skin disease virus <i>Mycoplasma capricolum</i> <i>Mycoplasma mycoides</i> Newcastle disease virus Peste des petits ruminants virus Rinderpest virus ^a Sheep pox virus Swine vesicular disease virus
	U.S. Department of Agriculture plant protection and quarantine
	Select agents and toxins
	<i>Coniothyrium glycines</i> (formerly <i>Phoma glycinicola</i> and <i>Pyrenochaeta glycines</i>) <i>Peronosclerospora philippinensis</i> <i>(Peronosclerospora sacchari)</i> <i>Ralstonia solanacearum</i> <i>Rathayibacter toxicus</i> <i>Sclerophthora rayssiae</i> <i>Synchytrium endobioticum</i> <i>Xanthomonas oryzae</i>

^aTier 1 agent.

source of microbial surveillance and training programs for laboratorians, health care providers, and first responders. The ongoing need to maintain diligent surveillance and state-of-the-art response capabilities requires that all first responders and laboratory scientists be also trained in the best practices of biosafety.

Biological risk management

Bioterrorism can appear to be a natural disease outbreak, and a natural disease outbreak can initially appear as bioterrorism. As a result, the clinical laboratory scientist should be prepared to evaluate the clinical specimen through the lens of **biorisk**. The spectrum of biorisk extends from the naturally occurring disease outbreak (or pandemic) to the intentional misuse of infectious agents. Between these two possibilities are a potential reemerging infectious disease, an unintended

consequence of research, and a laboratory accident. Each of these events presents the clinical laboratory with unique challenges. Imagine the risk associated with processing an average day's number of specimens only to encounter a SARS-like virus or a tuberculosis (TB) agent that day. Although we should always practice good microbiologic technique, a thorough understanding of the risk each specimen presents is best practice for every laboratory scientist.

Biorisk management is the management of risk associated with potential accidental release or exposure of biological agents through the integration of policies, practices, facility design, and safety equipment to enable an organization to effectively identify, assess, control, and evaluate the biosafety and biosecurity risks inherent in its activities. Biorisk management is grounded in the hierarchy of controls in which minimization of the risk is achieved first through elimination or substitution of the risk-causing material or process. If this

is not possible, then the risk is attempted to be minimized using a combination of engineering, administrative, or protective equipment controls. These controls establish specific room design, personal protective equipment (PPE), and standard operating procedures that decrease the risk of personnel and environmental exposure to the agents processed in a laboratory. Thus biological risk management increases the effectiveness of biosafety and biosecurity practices.

Biosafety

Implicit in our discussion of bioterrorism is the concept that agents misused for this purpose are chosen specifically because of their virulence, incubation period, lack of immunity, and thus, ability to incite fear and panic. Therefore the medical laboratory scientist should have a thorough understanding and appreciation of the principles and best practices required to remain safe from such agents in the clinical laboratory. A detailed guidance document produced by the CDC and recommended for all microbiology laboratories for assistance with safe work practices, PPE, and required engineering controls can be found in the most current edition of *Biosafety in Microbiological and Biomedical Research Laboratories* (BMBL).

In sum, **biosafety** is the application of laboratory practice and procedure, specialized laboratory facilities, and safety equipment used when working with potentially infectious material to reduce the risk of a laboratory-acquired infection. Although it is true that the agents often thought to be selected for bioterrorism are lethal, many of the common pathogens passing through hospital laboratories today are sufficiently virulent to produce outbreaks when they are evaluated and tested outside a biological safety cabinet. Therefore it is important to follow the CDC, World Health Organization (WHO), and ASM recommended practice of completing a **biological risk assessment** before performing any work with potentially infectious materials. The risk assessment process is an opportunity for the laboratory scientist to review information about the microorganisms, protocols, antimicrobial susceptibilities, PPE, respiratory protection, and so on to ensure the safest working conditions and to mitigate the consequences of the biohazard.

Regardless of risk group, microbiologists adhere to several basic safety principles to reduce exposure and prevent cross-contamination. These “standard microbiological practices” include frequent handwashing and no eating, drinking, gum chewing, or application of cosmetics in the laboratory. Mouth pipetting should not be performed. Aerosol generation should be avoided in the open laboratory. Personnel should be fully knowledgeable about the microorganism and trained on the procedure. They should use PPE, based on risk assessment results, follow standard operating procedures to reduce guesswork, and perform appropriate decontamination of work areas.

Biosafety levels

Laboratories can be placed into one of four **biosafety levels (BSLs)** based on the microorganism(s) encountered and the procedures with which the microorganisms will be manipulated (Table 30.2). A BSL reflects the microorganism **risk group**, along with a combination of standard procedures and

Table 30.2 Recommended biosafety level practices

BSL	Safety practices
1	Standard microbiological practices
2	BSL-1 practice plus limited access, biohazard warning signs posted, “sharps” precautions, biosafety manual defining standard operating procedures, and any needed waste decontamination or medical surveillance policies
3	BSL-2 practice plus controlled access into the laboratory, decontamination of all waste exiting the laboratory, decontamination of laboratory clothing before laundering, and baseline serum antibody analysis specific to agents used in the laboratory
4	BSL-3 practices plus clothing change before entering the laboratory, showering on exiting the laboratory, and all material decontaminated on exiting the facility

BSL, Biosafety level.

techniques, safety equipment, and facilities designed to minimize the exposure of workers and the environment to infectious agents.

Microorganisms are categorized on an increasing scale of 1 to 4 based on the risk they pose to human health and safety. Risk group 1 organisms are not known to consistently cause disease in healthy adults. Risk group 2 organisms are associated with human diseases. These are the organisms most often evaluated as part of the disease identification process in the clinical microbiology laboratory. They are hazardous to the laboratorian should they be ingested, exposed to a mucous membrane, or enter the host by percutaneous injury. Risk group 3 organisms are also associated with human diseases and can cause serious and even fatal consequences. They are indigenous or exotic agents with the potential for aerosol transmission. Risk group 4 viruses (there are no risk group 4 bacteria yet) are dangerous and often exotic and pose a high risk of life-threatening human diseases. They are readily transmitted by aerosol or direct contact.

Importantly, the organism risk group does not automatically equate to the BSL of the laboratory in which it is manipulated. Because BSLs are defined according to the risk group of the organism that is studied or tested *and* the procedures used, some organisms can fall under more than one BSL category based on the extent of studies. For example, *Mycobacterium tuberculosis* is a risk group 2 microorganism in clinical laboratories that perform only direct examination of clinical specimens. As such, those examination studies can proceed at BSL-2. Laboratories that concentrate TB bacteria, manipulate cultures, and create an aerosolization risk should perform these procedures in a BSL-3 containment facility. It is important to remember that each higher level includes additional safety equipment, precautions, engineering, and work controls over the previous level.

As mentioned earlier, the original list of select agents likely to be used as weapons of bioterrorism was created in 1999. The Public Health Security and Bioterrorism Preparedness and Response Act of 2002 authorized Congress to create a regulatory body that held authority to create and enforce regulations dealing with select agents and toxins. The Federal Select Agent Program became that body. The CDC oversees select agents and toxins that cause disease

in humans, while the U.S. Department of Agriculture oversees the select agents and toxins that affect plants and animals. Agents that cause disease in people and animals are overseen by both agencies. The 2010 Executive Order 13546 required the Select Agent Program to streamline policies and tighten perceived security breaches. In 2012, the biological select agents and toxins list was reevaluated. Some agents were removed, while others were reassigned to a new tiered list based on (1) the potential for causing death, (2) endemicity in the United States (animal agents), and (3) the potential of producing quantities necessary for a high consequence event (see Table 30.1). **Tier 1 agents** that cause human disease include *Bacillus anthracis*, *Burkholderia mallei*, and *B. pseudomallei*; *Clostridium botulinum* neurotoxin-producing strains; *Francisella tularensis*; *Yersinia pestis*; EBOV; Marburg virus (MARV); and variola viruses.

Case check 30.4

B. anthracis falls under the biological agent category because of its natural occurrence in the environment and ability to cause disease and death. It can be easily and inexpensively cultivated and has been demonstrated to cause disease via the respiratory tract, gastrointestinal (GI) tract, and abraded skin. Because *B. anthracis* can produce spores, this organism can survive very harsh conditions and remain infectious. The Case in Point is an example of an individual who was infected by inhalation of anthrax spores and became ill shortly thereafter.

Agents of bioterror

Bacillus anthracis

B. anthracis is an aerobic, gram-positive rod that produces endospores. Anthrax, the disease caused by *B. anthracis*, is primarily a disease of herbivores affecting mainly cattle, sheep, and goats. It has also been termed *wool sorter's disease* or *rag picker's disease* as the spores trapped in wool or on pelts can be transmitted to humans. Anthrax derives its name from the Greek word for coal, based on the characteristic black, necrotic, ulcerative skin lesion (eschar) noted in patients with cutaneous anthrax. Infected animal carcasses seed the soil with vegetative bacilli.

A key feature of *B. anthracis* is its ability to sporulate in response to poor growth conditions, imparting resistance to biological extremes of heat, cold, pH, desiccation, chemicals, irradiation, and other adverse conditions. These organisms can survive for years until taken up by another host, when germination and multiplication can resume. Natural infections in humans usually occur because of interactions with animals or contaminated animal products. Other than infections caused by the mailed spores, anthrax infections in humans have been rare (one or two cases per year) in the United States for at least the past century. Most cases have been cutaneous. Until the anthrax attacks in 2001, the last reported case of **inhalation anthrax** occurred in 1978. Twenty-two cases of anthrax were caused by the 2001 terrorist attack. In 2004, *Bacillus cereus* biovar *anthracis* was recognized as causing an anthrax-like disease in gorillas and chimpanzees in Cameroon and Côte d'Ivoire. Even though as of 2021 no human infections by this

agent have been identified, it was still added to the Tier 1 Select Agent list in 2016. The CDC determined that this bacterium has all the virulence determinates and biothreat potential of *B. anthracis*.

Clinical manifestations

Once inside the host, *B. anthracis* spores are phagocytosed by macrophages. Spores germinate into vegetative cells, capable of producing toxins and resisting phagocytosis. *B. anthracis* produces two plasmid-encoded virulence factors: a three-protein A/B exotoxin (A₂B) and a polypeptide capsule. The plasmid pX01 encodes genes for three specific components of the anthrax toxin: lethal factor (LF), edema factor (EF), and protective antigen (PA). Both LF and EF become biologically active toxins when combined with PA. The plasmid pX02 encodes genes to form a polypeptide (poly-D-glutamic acid) capsule made by the vegetative bacteria, which inhibits phagocytosis.

Human cases of anthrax can be distinguished by their method of exposure, which are typically occupational in nature, industrial (individuals who process bones, hides, wool, or other animal products), and nonindustrial (farmers, veterinarians, butchers). There are three main routes of exposure: cutaneous, ingestion, and inhalational. Worldwide, cutaneous anthrax is the most common form of the disease, accounting for about 95% of all cases. The disease occurs after a minimum of approximately 8000 spores are introduced into cuts or abrasions of human skin. After 1 to 6 days, a papule appears and then progresses to a 1- to 2-cm vesicle within the next 48 hours. When the vesicle ruptures, the resulting necrotic lesion develops into the characteristic black **eschar**, which can be edematous, but is usually painless (Fig. 30.4). The lesion usually heals without incident. The patient may or may not have systemic symptoms. Mortality associated with cutaneous anthrax is less than 1% with appropriate antimicrobial therapy but can be as high as 20% without treatment.

Oral or GI anthrax occurs when anthrax spores are introduced into the tissues of the GI tract, often by eating meat containing anthrax spores. There are two distinct manifestations: oropharyngeal, in which the lesion is localized in the



Fig. 30.4 Cutaneous anthrax lesion on the skin of the forearm. (Courtesy James H. Steele and Centers for Disease Control and Prevention, Atlanta, GA.)

buccal cavity, tonsils, or posterior oropharyngeal wall, and GI, which can occur anywhere within the remaining GI tract. After an incubation period of 2 to 6 days, the patient may experience sore throat, difficulty swallowing, swollen lymph nodes (oropharyngeal anthrax), and nausea and vomiting, rapidly progressing to fever, bloody diarrhea, ascites, and often sepsis (GI anthrax). Intestinal anthrax has a variable mortality rate because of the delayed initiation of appropriate therapy, often because of the nonspecific early symptoms.

Inhalational anthrax occurs when a patient inhales *B. anthracis* spores, typically from an animal or environmental source. Person-to-person transfer is uncommon. Inhalational anthrax is the form of anthrax most likely to be seen in a bioterror event because of the aerosol dispersion of anthrax spores. Spores that are 1 to 5 μm in size can reach the alveoli of the lung. Alveolar macrophages phagocytose the spores, which are transported to the mediastinal lymph nodes. After an incubation period of 1 to 6 days, patients develop nonspecific flu-like symptoms with fever, malaise, and fatigue as vegetative *B. anthracis* cells invade the bloodstream from the lymph nodes. Respiratory symptoms become more severe within the next few days as the patient experiences acute respiratory distress.

The differential diagnosis includes other causes, but the chest x-ray of an anthrax patient shows **mediastinitis** (Fig. 30.5). Pneumonia does not usually result from pulmonary infection. Data from the accidental spore release in Sverdlovsk, Russia, in 1979 suggest that pulmonary anthrax can result from inhalation of 1 to 10 viable spores, leading to a mortality rate of almost 100% without treatment. Because of rapid diagnosis and effective treatment, the mortality rate of inhalational anthrax in 2001 in the United States was less at 45% (5 of 11).

B. anthracis has always been listed as a select agent likely to be used for bioterrorism. The accidental release of spores

at Sverdlovsk, the largest documented outbreak of human inhalation anthrax, demonstrated the potential for misuse. Because most cases are associated with cutaneous exposure, whenever an individual develops a GI or inhalational form of disease, it is important to rapidly distinguish between a natural exposure and a deliberate release. Recent cases of naturally acquired noncutaneous anthrax have increased concerns. Some notable cases include drum makers who developed inhalational anthrax from working with contaminated animal hides, an individual who ingested airborne spores while participating in a community-wide drumming ceremony and developed GI anthrax, and an outbreak of anthrax septicemia or wound infections in the United Kingdom among intravenous (IV) drug users injecting contaminated drugs. Further investigations into these cases found only individuals or similar groups of people who became infected via natural causes. However, the massive exposures that can be created in deliberate release scenarios cannot be emulated by nature.

Specimen collection and preparation

The specimen of choice for cutaneous anthrax diagnosis is vesicular fluid from fresh vesicles. Swabs can also be used to collect material from under the edge of the crust of the eschar. The specimen of choice for diagnosis of patients with GI anthrax is blood, before antimicrobial therapy. Ascites fluid is used for cultures and real-time polymerase chain reaction (rtPCR). Stool and rectal swabs can also be cultured and tested by rtPCR. The specimen needed to diagnose inhalation anthrax is also blood. Because inhalation anthrax usually does not result in pneumonia, and vegetative bacteria are not likely to be found in the lungs, the usefulness of sputum specimens is less than that of blood. Cerebrospinal fluid (CSF) from patients with meningeal signs is also useful for culture and rtPCR.

Direct examination and initial culture

Smears from lesion fluid, eschar, blood, CSF, or tissues may show large, square-ended, boxcar-like gram-positive bacilli. There may be evidence of a capsule around the cells. Spores most likely will not be evident in direct smears from clinical specimens because spores form only in poor nutritional environments.

In aerobic culture, *B. anthracis* will yield large (2 to 5 mm) nonhemolytic colonies on 5% sheep blood agar (SBA). Colony growth can be rapid and occur in as little as 8 hours. Mature colonies have a ground-glass appearance. The colony often has feathery projections coming from the margin, referred to as *Medusa-head* colonies (Fig. 30.6). Colonies also have a tenacious consistency, which—when teased with a loop, for example—will form peaks, like whisked egg whites (Fig. 30.7). The gram-positive bacilli (Fig. 30.8) can show oval endospore formation in late-stage cultures (Fig. 30.9). The spores are central or subterminal and do not cause the bacterial cell to swell.

Tests for presumptive identification

According to CDC protocols, a presumptive identification of *B. anthracis* can be made if an isolate has the following characteristics: (1) aerobic growth and (2) nonhemolytic colonies 2 to 5 mm in diameter containing catalase-positive, nonmotile,



Fig. 30.5 Chest radiograph of a patient who worked in a goat hair processing mill. Note the widened mediastinum, often seen with inhalation anthrax. (Courtesy Arthur E. Kaye and Centers for Disease Control and Prevention, Atlanta, GA.)



Fig. 30.6 “Medusa head” appearance of *Bacillus anthracis* nonhemolytic colonies, with feathery projections from the edges at 24 hours. (Courtesy Edward F. Keen, Brooke Army Medical Center, San Antonio, TX.)

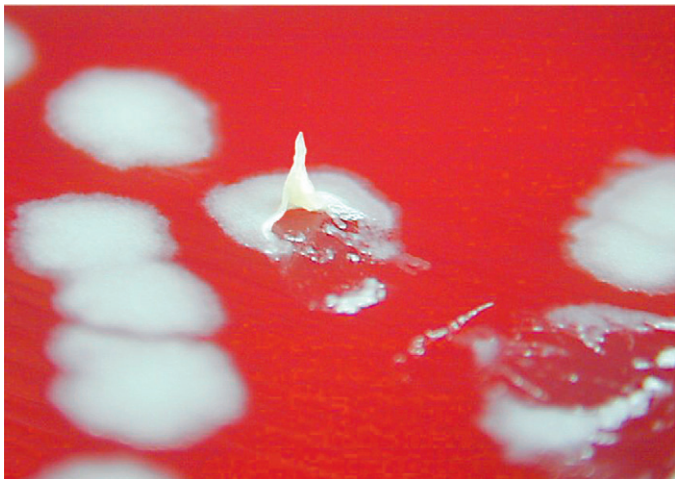


Fig. 30.7 Tenacious consistency (stiff egg white appearance) of *Bacillus anthracis* colonies on sheep blood agar plate at 24 hours. (Courtesy Larry Stauffer, Oregon State Public Health Laboratory, Hillsboro, OR, and Centers for Disease Control and Prevention, Atlanta, GA.)

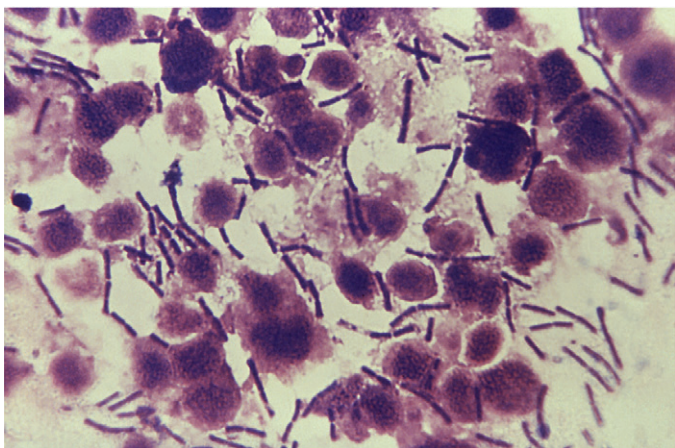


Fig. 30.8 Gram-stained smear of *Bacillus anthracis* from a patient specimen ($\times 1000$). (Courtesy Centers for Disease Control and Prevention, Atlanta, GA.)

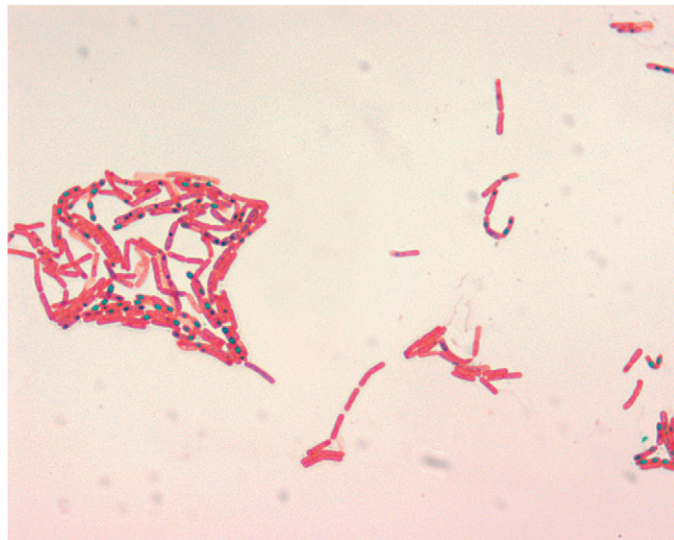


Fig. 30.9 *Bacillus anthracis* culture (malachite green spore stain, $\times 1000$). (Courtesy Larry Stauffer, Oregon State Public Health Laboratory, Hillsboro, OR, and Centers for Disease Control and Prevention, Atlanta, GA.)

large gram-positive bacilli recovered from lesions, blood, CSF, or lymph nodes. Oval, central to subterminal spores may be present. Other bacilli, such as *Bacillus cereus* var. *anthracis* and *Bacillus cereus* var. *mycoides*, can also have this profile. *Bacillus megaterium* is often confused morphologically for *B. anthracis*. Organisms with a presumptive identification of *B. anthracis* must be sent immediately to an LRN reference laboratory for confirmatory identification. The recovery of *B. anthracis* in inhalational or GI anthrax is a sentinel event in the United States.

Case check 30.5

According to the requirement of the Select Agent Program, the clinical laboratory in the Case in Point that isolated the *B. anthracis* from the hospitalized patient should ship all isolates of the organism to its state laboratory and destroy such isolates within 7 days of confirmation by that state laboratory.

Burkholderia species

The genus *Burkholderia* contains two species considered to be potential agents of bioterrorism: *B. mallei* and *B. pseudomallei*. *B. mallei* causes the disease known as **glanders**; *B. pseudomallei* is the causative agent of **melioidosis**. *B. mallei* has disappeared from most regions of the world. However, it is enzootic in Asia and several eastern Mediterranean countries, where environmental isolates have been recovered from soil and water. Sporadic cases among those whose occupations involve contact with infected horses, mules, and donkeys and in laboratory scientists have been reported in the Western Hemisphere. Glanders manifests itself in three forms: (1) a chronic pulmonary form with cough, producing a mucopurulent sputum; (2) a form characterized by abscesses of the skin, subcutaneous tissues, and lymphatics, known as *farcy*; and (3) an acute septicemic form that rapidly progresses from

fever, chills, and prostration to death, within 7 to 10 days. The disease does not appear to be readily transmitted from person to person, although two human cases of transmission by sexual and direct contact have been reported in the literature.

Melioidosis, caused by *B. pseudomallei*, is a disease similar to glanders but found in different parts of the world. *B. pseudomallei* is endemic to Southeast Asia (particularly Thailand), the South Pacific, Africa, India, and the Middle East. Although not as common, the organism has been isolated from patients in Mexico, Panama, Peru, and Haiti. The organism has also been recovered from patients in Hawaii and Georgia. Approximately one to five cases are reported in the United States each year and are primarily travel related. This organism is a saprophyte found commonly in soil and water.

Clinical manifestations

The infectious doses of *B. mallei* and *B. pseudomallei* are not known but are thought to be low because of the ease of infectivity. The incubation periods are variable, ranging from 1 to 14 days. Disease in humans depends on the route of transmission. Symptoms of infection include fever, myalgia, headache, and chest pain. Infection can result in localized cutaneous lesions, septicemia, and pneumonia. Bloodstream infections are often fatal. Although a human case of laboratory-acquired infection with *B. mallei* was documented in Maryland in 2000, no naturally acquired cases of human infection with *B. mallei* have been recorded in the United States since 1945.

Transmission of *B. pseudomallei* is usually related to contact with soil and water, direct contact with infected animals, or inhalation. Rare, documented cases of person-to-person transmission, primarily through blood and body fluids, have also been reported. Clinical infection is similar to that of glanders in humans. If the patient is infected via direct contact and bacterial entry occurs through broken skin or mucous membranes, the clinical presentation is a localized nodule. However, hematogenous dissemination can also occur, resulting in acute sepsis. Bloodstream dissemination of the organism can result in a chronic form of melioidosis, with lesions forming at multiple internal organs. If the organism enters via inhalation, the clinical presentation can range from mild bronchitis to severe lower respiratory tract infection.

More recent case reports have identified latent infections in which clinical infection manifests itself between the fourth and fifth decades of life in patients with underlying complications of diabetes mellitus, excessive alcohol consumption, chronic renal failure, and chronic lung disease. In the United States, many of the reported cases were noted in war veterans who had served in Vietnam years earlier and developed signs and symptoms of disease, likely because of a waning immune status. This condition has been termed the *Vietnamese time bomb*, indicating that the patient was likely infected years earlier, but because of age and risk factors noted, the disease is a reactivation of a latent infection.

Specimen collection and preparation

Because laboratory transmission is a high risk with culture and manipulation of these organisms, health care providers suspecting infections by *B. mallei* and *B. pseudomallei* should notify the clinical laboratory when submitting specimens for

testing. Specimens appropriate for submission to detect either of these isolates are blood, bone marrow, sputum, bronchoalveolar lavage, abscess material, urine, and serum. Automated identification systems should not be used if *Burkholderia* spp. are suspected.

Direct examination and initial culture

Direct Gram-stained smears of aspirates and respiratory secretions will reveal gram-negative coccobacilli for *B. mallei* and small gram-negative bacilli for *B. pseudomallei*. These organisms will also be noted on direct smear examination of blood cultures from these patients. If *B. mallei* or *B. pseudomallei* is suspected, cultures are incubated at 35°C in 5% carbon dioxide (CO₂) for up to 5 days. *B. mallei* grows as small, circular, butyrous colonies after 24 to 48 hours of incubation on SBA. If held longer, the colonies will gradually convert to a dry, wrinkly appearance, resembling those of *Pseudomonas stutzeri*. *B. mallei* and *B. pseudomallei* typically grow on MacConkey (MAC) agar and SBA (Fig. 30.10). *B. pseudomallei* and *B. mallei* colonies resemble each other. *B. pseudomallei* may produce a smell like that of soil; however, smelling bacterial colonies with the plate open is hazardous and should never be attempted.

Tests for presumptive identification

Gram-negative coccobacilli or small gram-negative bacilli with poor growth at 24 hours forming larger nonpigmented gray colonies at 48 hours that are indole negative, nonmotile, catalase positive, and resistant to polymyxin B or colistin are presumptively identified as *B. mallei* and must be submitted immediately to the nearest LRN reference laboratory. Gram-negative coccobacilli or small gram-negative bacilli that exhibit poor growth at 24 hours but form larger nonpigmented gray colonies at 48 hours, begin to wrinkle after 48 hours, grow on MAC agar, are oxidase positive and indole negative, and are resistant to polymyxin B or colistin cannot be ruled out as *B. pseudomallei*. An isolate with these characteristics should be referred to an LRN reference laboratory for confirmation.



Fig. 30.10 Colonies of *Burkholderia pseudomallei* (causative agent of melioidosis) on sheep blood agar incubated at 37°C for 72 hours. (Courtesy Dr. Todd Parker and Centers for Disease Control and Prevention, Atlanta, GA.)

Clostridium botulinum neurotoxin

Clinical manifestations

Botulinum toxin is a neurotoxin primarily produced by *Clostridium botulinum*, although other clostridia, including *C. butyricum* and *C. baratii*, can also produce the toxin. Seven main types of toxins, designated A to G, are well known, although new types are occasionally found. The types commonly implicated in human disease are A, B, E, and rarely F.

C. botulinum spores can be recovered from soil specimens throughout the world. Although rare in the United States, naturally occurring botulism cases do occur. There are four typical manifestations of disease: foodborne botulism, infant botulism, intestinal botulism caused by bacterial colonization in children and adults, and wound botulism. The CDC has kept national botulism surveillance since 1973; an average of 163 U.S. cases of botulism were reported annually between 2001 and 2020, with most cases occurring in infants. Foodborne botulism occurs when *C. botulinum* spores survive in poorly washed and improperly preserved or canned food products. The spores germinate under anaerobic conditions, inside the food container, into vegetative cells producing neurotoxin. Consumption of the toxin-tainted food leads to disease; this is an example of intoxication with bacterial toxin. The most commonly associated foods are home-canned vegetables, meats, smoked or raw fish, and honey or corn syrup.

Infant botulism is the most commonly recognized form and typically affects infants between 3 weeks and 6 months of age. Infection occurs when the child ingests a spore via soil exposure or contaminated foods, such as raw honey. In this case, a true infection occurs when the spore germinates, and vegetative bacteria colonize the intestinal tract. Infants who are formula fed are most prone to this because their still maturing gut microbiota is unable to fend off this infection. Adult-onset botulism, resulting from infection, is similar to the infant form; their gut microbiota is also unable to prevent infection, typically because it has been compromised by administration of antimicrobial agents or surgery. Wound botulism occurs when a spore enters an open wound, converts to a vegetative cell, and proliferates, producing the toxin. This type of infection has most commonly been recognized in IV drug users.

Adults are more likely to have intoxication botulism. The period between exposure and symptoms differs depending on the route of exposure, the form of disease (i.e., intoxication or infection), and the amount of toxin. For example, an ingestion toxemia might take from 2 hours to 8 days; the average, however, is 18 to 36 hours. Fever is absent, and patients often develop trouble speaking, swallowing, and seeing (blurred vision). The toxin causes a descending, progressive, bilateral muscle weakness resulting in flaccid paralysis, ultimately ending with respiratory failure and death. Disease mortality can be 25% but more typically is only 5% to 10% when medical intervention, such as mechanical ventilation or intubation, is provided promptly. The toxin irreversibly binds to neurotransmitters, so antitoxin and a toxoid vaccine should be administered immediately if botulism is suspected. Some hallmark features of infant botulism include slowed breathing, poor feeding, constipation, weak cry, loss of head control, and essentially a floppy appearance (Fig. 30.11).

Botulinum toxin is the most lethal of all biological agents; the lethal dose that kills 50% of test animals is **1 ng per kilogram of body weight**. If a single gram of crystalline toxin



Fig. 30.11 A 6-week-old infant with botulism caused by ingestion of *Clostridium botulinum* spores, noted by loss of muscle tone in the head and neck areas. (Courtesy Centers for Disease Control and Prevention, Atlanta, GA.)

were optimally dispersed over a city, it is estimated that there would be over 1 million deaths. It has been further estimated that based on its lethal dose, **8 oz would be enough to kill nearly every human on the planet**. Wound-associated cases are not likely to be associated with a terrorist attack; it is more likely that larger-scale inhalation or foodborne ingestion of the toxin would be a biocrime.

Specimen collection and diagnosis

Because of its rapid effects, an initial diagnosis of botulinum toxin exposure should be based on symptoms, as opposed to laboratory data. Thus treatment should begin before laboratory confirmation. The LRN stresses that botulism is a public health emergency and that local and state public health officials should be notified upon diagnosis. Most local hospitals are not equipped to process specimens for the detection of botulinum toxin; therefore specimens should be submitted to the nearest LRN reference laboratory. Specimens appropriate for referral include feces, gastric aspirate or vomitus, serum, tissues or exudates, and suspected food specimens. Environmental specimens should also be submitted to the LRN reference laboratory, if possible.

Diagnosis of botulism can be confirmed by the detection of organisms in culture or botulinum toxin in specimens. The traditional method that confirms botulinum toxin and identifies the serotype is a mouse toxicity assay. Some enzyme immunoassay tests are sensitive enough to detect the minute amounts of residual toxin found in the respiratory tract of affected patients. LRN reference laboratories also use rtPCR to confirm the detection of botulinum toxin.

Ebola virus and Marburg virus

EBOV and MARV are zoonotic pathogens that cause severe hemorrhagic fever in humans. They are members of the virus family *Filoviridae*, sharing some common

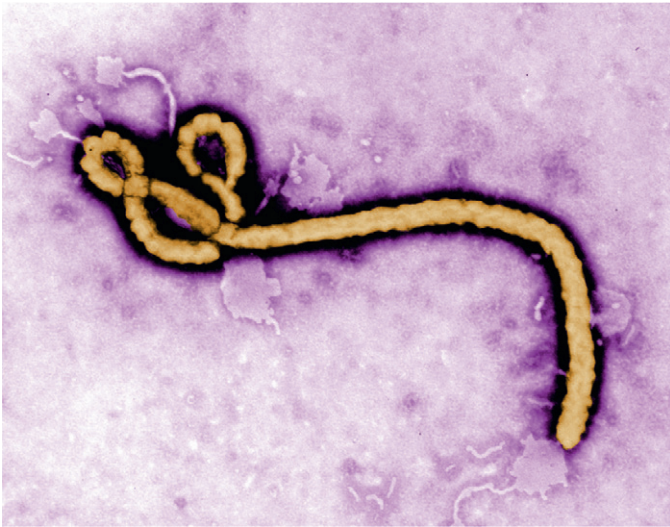


Fig. 30.12 Transmission electron micrograph of an Ebola virus virion (causative agent of hemorrhagic fever) by negative stain technique ($\times 100,000$). (Courtesy Dr. Fred Murphy and Centers for Disease Control and Prevention, Atlanta, GA.)

characteristics. By electron microscopy, the filoviruses have a distinctive ropey appearance that makes them easily recognizable. They are pleomorphic, filamentous particles that may be coiled, toroid, or branched; thus they can assume different shapes, such as a shepherd's crook, a number 6, and a letter U (Fig. 30.12). They are relatively small (80 to 1200 nm, larger when grown in tissue culture), enveloped, single-stranded ribonucleic acid viruses that require an animal host as a reservoir. The natural reservoirs of these viruses have still not been determined. However, humans are not the natural reservoir for either of these viruses; rather, humans become infected when they encounter the natural host or another animal infected with the virus. Importantly, subsequent person-to-person transmission can occur without strong public health measures in place to contain the disease. Contact with nonhuman primates appears to be instrumental in several EBOV outbreaks, but the primates are not the natural reservoir. Some evidence indicates a role for bats in the persistence of EBOV in the environment. Airborne or droplet transmission is not considered a common natural route of transmission of EBOV and MARV. Direct contact with infected body fluids seems to be the primary mode of transmission; therefore effective use of PPE and "contact precautions" generally stops the transmission chain.

There are six known species of the genus *Ebolavirus*; five of them are well characterized, and four cause human disease. They are (1) *Zaire ebolavirus* (ZEBOV), the cause of most outbreaks, 70% to 90% fatality rate; (2) *Sudan ebolavirus* (SUDV), 53% to 68% fatal; (3) *Reston ebolavirus* (RESTV), a nonhuman pathogen; (4) *Tai Forest ebolavirus* (TAFV), formerly *Cote d'Ivoire ebolavirus*, unknown human pathogenicity; (5) *Bundibugyo ebolavirus* (BEBOV), 34% fatal; and (6) *Bombali ebolavirus* (BOMV), unknown human pathogenicity. EBOV was first recognized during epidemics in Sudan and the Democratic Republic of the Congo (formerly Zaire) in 1976. EBOV has made a deadly appearance nearly every year since then. Significantly, 32,377 cases of EBOV disease were reported

from 2014 until the end of 2020, with an average mortality rate of 43%. Clearly the filoviruses present a huge challenge when preparing for bioterrorism because of the frequency with which these highly pathogenic viruses erupt into the world, the fatality rates that they produce, and the fear that they command.

Marburg virus disease was first described in 1967 during concurrent outbreaks in Marburg and Frankfurt (Germany) and Belgrade (Yugoslavia, now Serbia) resulting from laboratory-acquired transmission from African green monkeys imported from Uganda. Two viruses that share more than 90% nucleotide homology cause Marburg disease. Both are of the genus and species *Marburg marburgvirus*. In 2009, the Egyptian rousette, or Old-World fruit bat, was found to harbor both strains of *M. marburgvirus*, implicating it as a potential reservoir host. Since its discovery, at least 10 outbreaks of Marburg disease were recorded through 2014, including two (1988 and 1990) in Koltsovo (in the former Soviet Union) purportedly resulting from laboratory accidents. There are two MARVs that cause Marburg virus disease: Ravn virus (RAVV) and MARV. Because the genomes of both viruses diverge by <10% from the prototype *Marburg marburgvirus*, they are considered to both be strains of MARV.

The U.S. Food and Drug Administration (FDA) licensed a vaccine for the *Zaire ebolavirus* in 2019. It is a replication-competent, live, attenuated, recombinant vesicular stomatitis virus (rVSV) vaccine. This vaccine has yet to be tested against other filoviruses. There are also two biologic drugs licensed by the FDA to treat *Zaire ebolavirus*. The first drug is a combination of three monoclonal antibodies. The second drug is a single monoclonal antibody. They have yet to be approved for disease caused by other filoviruses. Experimental small drug molecules for both EBOVs and MARVs are in development.

Clinical manifestations

Clinical manifestations of these infections differ depending on several factors, including viral strain. In general, the infectious dose for EBOV and MARV is 1 to 10 infectious particles. The incubation period for most of these infections is 4 to 22 days, after which the patient may experience some or all of the nonspecific symptoms, such as fever, rash, myalgia and arthralgia, nausea, conjunctivitis, diarrhea, and central nervous system symptoms (headache, meningitis). Hemorrhagic fever is not as common a manifestation as with other filoviruses. Infections can include a varied degree of bleeding disorders, ranging from disseminated intravascular coagulation (DIC), petechiae, and hemorrhage of the mucous membranes to conjunctivitis, blood-tinged urine, and blood-tinged vomitus. Generally, patients infected with these filoviruses might be febrile and have a maculopapular rash, bleeding, and DIC.

Specimen collection and preparation

Specimens should not be collected from patients with suspected EBOV or Marburg disease or processed until after consultation with public health officials. Specimens appropriate for the diagnosis of EBOV or MARV disease include serum, heparinized plasma, whole blood, respiratory aspirates, tissue, and urine.

Francisella tularensis

Tularemia is a plaguelike, zoonotic disease caused by *F. tularensis* that was first isolated in 1911, when it was found to be the cause of an outbreak in ground squirrels in Tulare County, California. *F. tularensis* is distributed across the entire Northern Hemisphere. Natural infections occur annually in various parts of the world. In the United States, 2537 cases were reported from 2007 to 2020.

Human infections usually occur by accidental contact with the organism, via arthropod bite, by direct contact with infectious materials, by ingestion of contaminated food or water, or by inhalation of infective aerosols. Hunters or outdoor enthusiasts typically have a higher rate of infection because of contact with infected carcasses and animals or vectors, including ticks, mosquitoes, and flies. Because of the numerous ways this infection can be transmitted, tularemia has numerous nicknames to match, including *deer fly fever*, *rabbit fever*, *Ohara fever*, *market men's disease*, and *water trapper's disease*. The most common U.S. reservoirs are several species of ticks (e.g., *Ixodes*, *Amblyomma*, *Dermacentor*, *Haemaphysalis*) and numerous other arthropods. The most common mammal associated with tularemia in the United States is the rabbit.

The infectious dose for the bacterium causing cutaneous or inhalational disease in humans is as low as 10 to 100 organisms. Natural infections resulting from aerosolized *F. tularensis* have been described, often from handling contaminated hay, mulch, water, or infected carcasses. An interesting case of pneumonic tularemia was recorded when two adolescents became ill after mowing a grassy area. The high degree of infectivity, incapacitating nature, and natural occurrence of the organism has marked it as a potential biological weapon. The WHO estimates that 50 kg of organism could infect 125,000 people in a city of 5 million people and could cause up to 19,000 deaths.

Clinical manifestations

Tularemia has different clinical manifestations. The most usual clinical presentation is ulceroglandular disease, during which the organism is transported to the local lymph nodes and disseminated throughout the bloodstream. The naturally occurring infection results from inoculation of the organism into the dermis of the patient (Fig. 30.13). Inoculation occurs via the bite of a *F. tularensis*-infected arthropod or from handling infectious materials. The latter is common among those engaging in outdoor sports. The ulceroglandular form has an incubation period ranging from 2 to 10 days, with an average of 3 to 4 days, after which patients become abruptly febrile, with chills, headaches, cough, and chest pain. Patients develop lesions at the site of entry, and these are slow to heal and can persist for weeks to months.

The glandular form occurs when organisms are transported to lymph nodes, causing lymphadenopathy, similar to the buboes of bubonic plague. Hematogenous dissemination can occur, resulting in multiorgan seeding. Even without antimicrobial therapy, patients who progress to this point rarely die of this form of the disease. Symptoms resolve over a period of months, but the patient experiences malaise, anorexia, fatigue, and weight loss.

Less commonly, patients develop oculoglandular, oropharyngeal, GI (on ingestion of contaminated materials), or inhalational disease. In the event of inhaling an aerosol of *F. tularensis*, patients develop pneumonic or



Fig. 30.13 Poorly healing ulcerative lesion of tularemia on the thumb. (Courtesy Dr. Thomas Sellers, Emory University, and Centers for Disease Control and Prevention, Atlanta, GA.)

inhalational tularemia. Fever and symptoms of a lower respiratory tract infection, essentially a flulike disease, can rapidly progress to dyspnea, hemoptysis, sepsis, meningitis, and shock. Chest radiography will reveal a widened mediastinum, similar to that seen with inhalation anthrax. Natural cases of inhalational tularemia do occur, usually in rural areas. Mortality rates of untreated inhalational tularemia can be 30% to 60%. In the United States, tularemia has an overall mortality of about 2%.

Direct examination and initial culture

Blood culture specimens are collected from patients with fever. If lesions are present, biopsy or culture specimens from the leading margin of the lesion can be collected and submitted for culture and direct examination. If the clinical presentation or patient history is consistent with tularemia, the laboratory must be notified before the submission of specimens. Specimens should be handled in a biological safety cabinet, and procedures generating aerosols should be performed with BSL-3 safety practices. Several incidents of laboratory-acquired infections have been recorded as workers processed specimens and cultures on an open bench, only later identifying the organism through biochemical testing or PCR.

Tests for presumptive identification

On Gram-stained smears, *Francisella* appears as tiny, pleomorphic, gram-negative coccobacillus (Fig. 30.14). *Francisella* is fastidious, requiring the amino acid cysteine for optimal growth. The organism grows well on media supplemented with cysteine, such as chocolate agar (Fig. 30.15A), modified Thayer-Martin medium, cysteine heart agar, and buffered charcoal-yeast extract agar (see Fig. 30.15B–D). *F. tularensis* grows very slowly, so colonies may not be visible on solid media until after 36 to 48 hours of incubation. Growth may be stimulated with increased CO₂ incubation. *F. tularensis* will not grow at all on MAC agar or eosin-methylene blue agar.

Pleomorphic, poorly counterstaining, gram-negative coccobacilli from blood cultures that do not grow well, if at all, on SBA should lead the laboratory scientist to consider

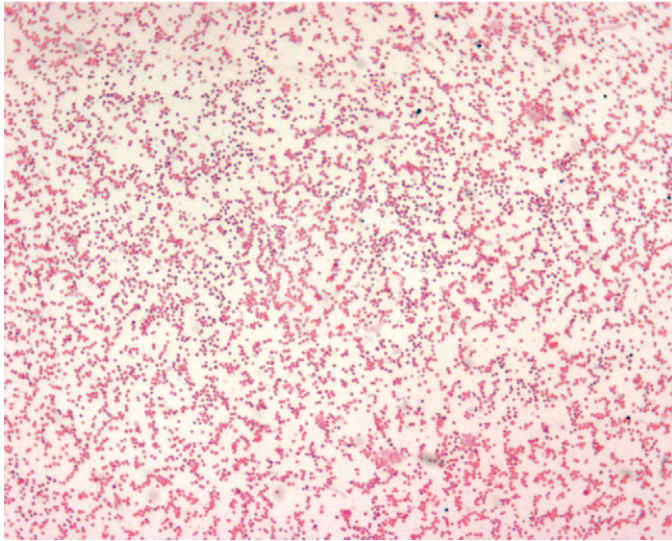


Fig. 30.14 Micrograph of *Francisella tularensis* demonstrating poorly staining, very tiny (0.2 to 0.7 μm) gram-negative coccobacilli (Gram stain, $\times 1000$). (Courtesy Larry Stauffer, Oregon State Public Health Laboratory, Hillsboro, OR, and Centers for Disease Control and Prevention, Atlanta, GA.)

F. tularensis. As a safety precaution, laboratory procedures must be done inside a biological safety cabinet until *F. tularensis* has been ruled out. Catalase and β -lactamase tests are positive for *F. tularensis*, whereas requirements for X and V factors, urease, and oxidase are negative. Isolates with these reactions are presumptively identified as *Francisella* and should be referred to an LRN reference laboratory for confirmatory identification, usually via PCR.

Variola virus

Variola virus is the causative agent of smallpox. The virus belongs to the family Poxviridae, genus *Orthopoxvirus*. These brick-shaped, double-stranded deoxyribonucleic acid (DNA) viruses measure approximately 200 nm in length (Fig. 30.16). Variola viruses are strictly characterized as risk group 4 because of the planetwide public health risk should they enter the unvaccinated population again. The clinical (sentinel) laboratory does not culture these viruses for identification, and there is no commercially available diagnostic kit for smallpox. The role of the sentinel laboratory is advisory to health care providers recommending specimen collection, packaging, and alerting the LRN.

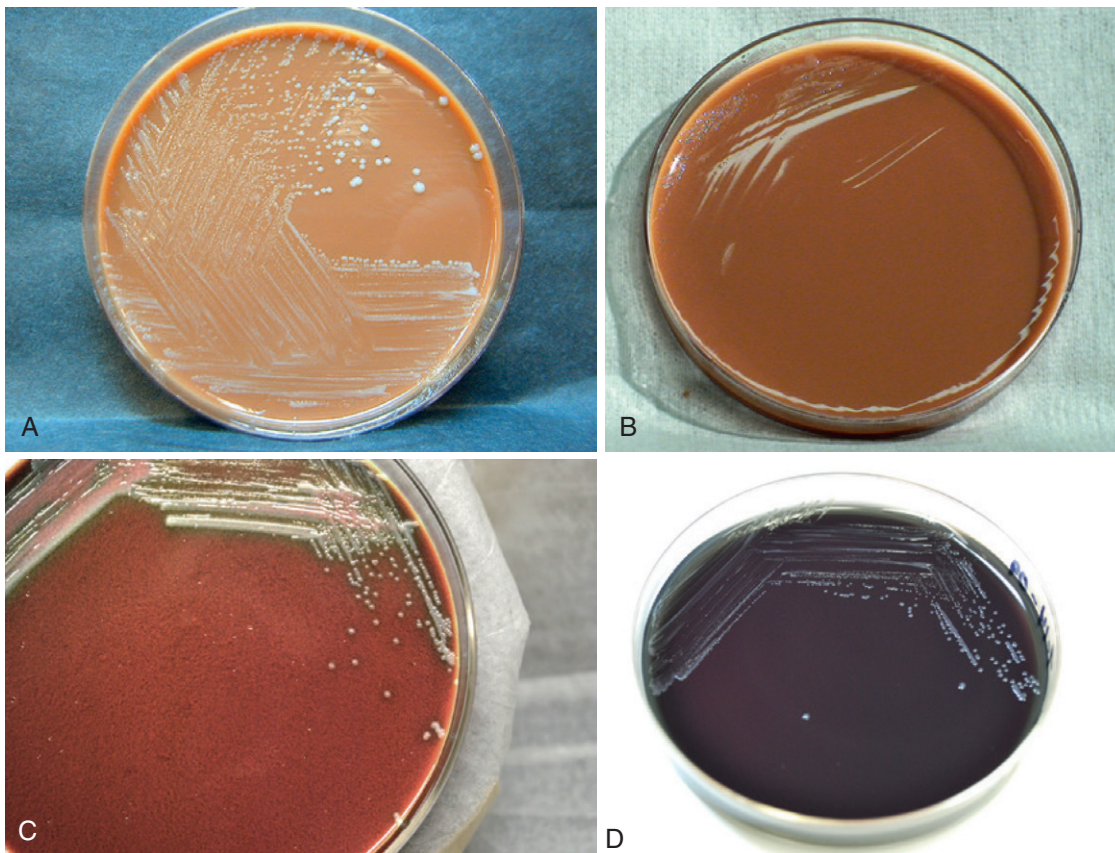


Fig. 30.15 Colonies of *Francisella tularensis* growing on supplemented chocolate agar (A), modified Thayer-Martin agar (B), cysteine heart agar with 9% chocolated blood (C), and buffered charcoal-yeast extract agar (D). (A, Courtesy Larry Stauffer, Oregon Public Health Laboratory, Hillsboro, OR; B, C, courtesy Amanda Moore, Dr. Todd Parker, and Audra Marsh; D, courtesy Megan Mathias and J. Todd Parker and Centers for Disease Control and Prevention, Atlanta, GA.)

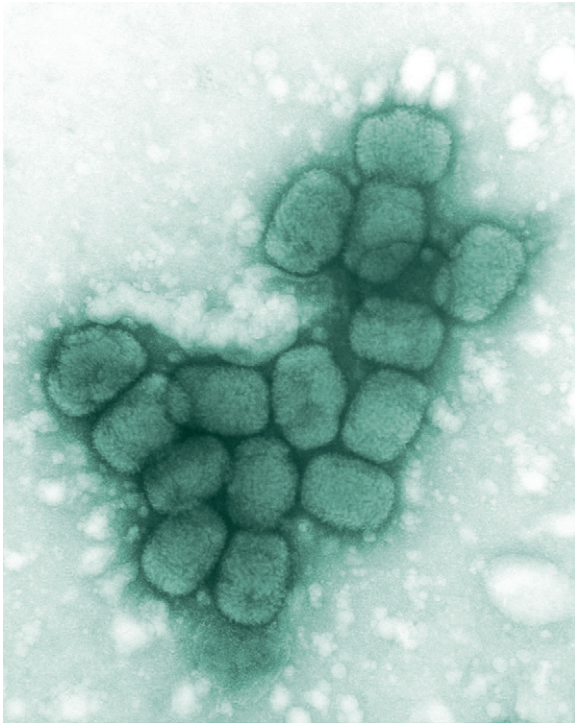


Fig. 30.16 Transmission electron micrograph of smallpox viruses by negative stain technique ($\times 100,000$). (Courtesy Dr. Fred Murphy and Centers for Disease Control and Prevention, Atlanta, GA.)

Clinical manifestations

Following a successful worldwide eradication program, the last naturally occurring case of smallpox (variola) was reported in 1977. The two major forms of the disease were **variola major** and **variola minor**. Although antigenically indistinguishable, variola minor caused a much less severe disease compared with variola major, with a mortality rate of about 1% compared with about 30% for variola major. Until the late 1800s, variola major predominated. Variola minor was first described in the late 19th century in Africa and the United States. This form of smallpox spread throughout North America and eventually reached Europe. The mortality rate of variola major was about 30% in unvaccinated patients but only about 3% in vaccinated patients. The vaccination consists of an attenuated vaccinia virus. The vaccine can rarely produce infection that is serious or even fatal.

Smallpox was typically transmitted through respiratory droplets via close contact with infected patients or by direct contact with scabs and virus-contaminated fomites. What is more concerning, however, is that the virus can also be spread by aerosol, and the dose required to initiate infection is quite low. Initially, the virus replicates within cells of the oropharynx or respiratory mucosa and later spreads to the regional lymph nodes and multiplies. The infected patient undergoes a transient asymptomatic viremia, resulting in hematogenous dissemination of the virus. About 8 to 10 days after exposure, the patient develops fever and malaise, presenting with a flu-like syndrome. Variola virions infect white blood cells, which are transported to small blood vessels under the dermis and oral mucosa. Oral lesions develop, and the patient is infectious at this point. The patient develops a light macular rash, starting on the head and arms and then spreading to the trunk



Fig. 30.17 Nepalese female demonstrating classic maculopapular rash of smallpox pustules over her entire body. (Courtesy David Bassett and Centers for Disease Control and Prevention, Atlanta, GA.)

and legs. The macular rash then progresses to a vesicular rash after 1 to 2 additional days.

One of the most characteristic signs of smallpox, differentiating it from other viral fevers and rashes, such as chickenpox (varicella), which is caused by a herpesvirus (varicella zoster virus), is the synchronous progression of the lesions; all lesions have almost the same appearance. Also, lesions are more heavily concentrated on the extremities because the virus prefers the cooler body temperature found in these locations. Another significant feature of smallpox is that the palms and soles are not spared, as they are with varicella. Vesicles continue to progress into pustules (Fig. 30.17). The pustules are unique in that they penetrate deeply into the tissue of the patient and are extremely turgid. It is at this stage—approximately 1 week after the start of the rash—that the patient is most likely to die. After about day 14 of the rash, the pustules start to scab and heal, leaving significant scarring. Once all scabs have fallen off, the patient is no longer infectious, whereas the scab material is highly infectious. The surviving patient would likely be immune to future infection by the virus. Variola minor disease is characterized by a less severe presentation, with a less dense rash.

Edward Jenner showed in 1796 that inoculation with the benign cowpox virus conferred resistance to smallpox. During the 19th century, vaccination programs began using the **vaccinia** virus instead of cowpox. In 1967, the WHO began a smallpox eradication program based on detection and vaccination, which ultimately ended smallpox infections globally. The last case of smallpox in the United States occurred in 1949, and the last documented case of natural smallpox was in Somalia in 1977. In 1980, the WHO made the triumphant declaration that the world was smallpox-free. All known stocks of variola were destroyed, except for two. One of the stocks remains at the CDC, and the second is maintained at the Russian State Research Center of Virology and Biotechnology in Koltsovo. There are lingering concerns that other undocumented stocks of smallpox virus may be currently maintained by other countries.

After the *B. anthracis* bioterror attack in the United States in 2001, several countries began stockpiling the smallpox vaccine. The method of vaccination is similar to the original variolation procedure, whereby a bifurcated needle containing a standardized amount of attenuated vaccinia virus is used to inoculate an individual (Fig. 30.18). In 2002 and 2003, a U.S. federal campaign to vaccinate health care workers against smallpox ended when many workers, concerned about

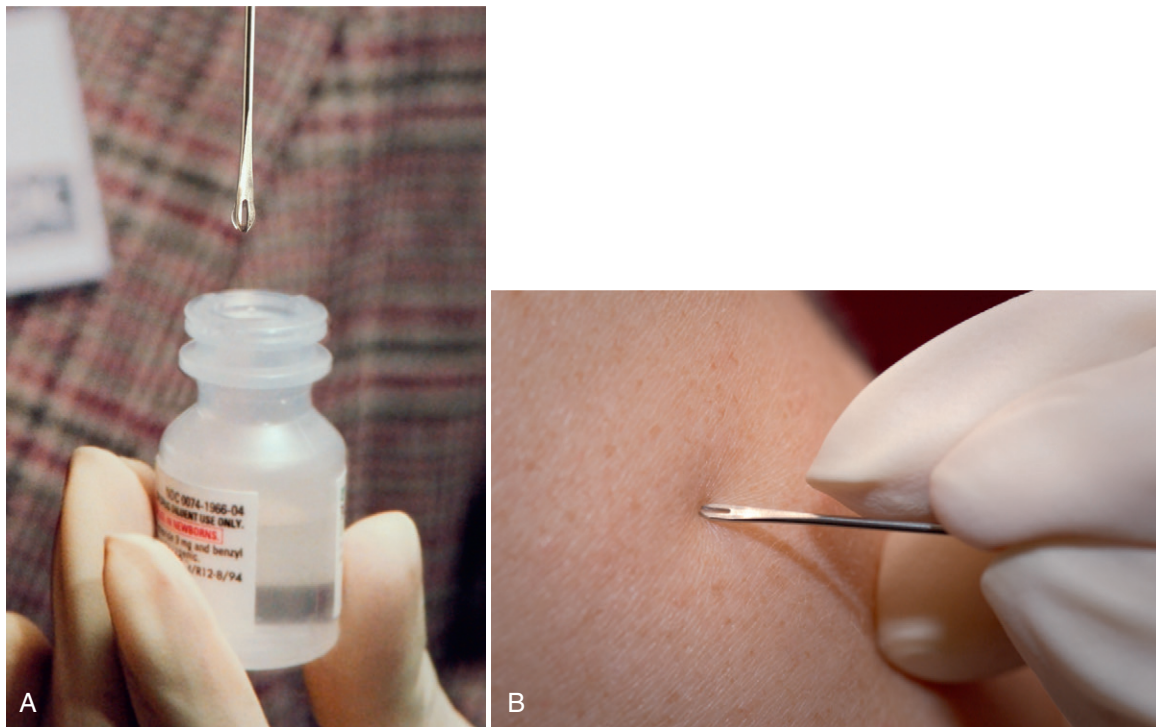


Fig. 30.18 Method of vaccination against smallpox using live vaccinia virus. A bifurcated needle holds a standard amount of virus (A), which is then inoculated into the individual's arm (B). (Courtesy James Gathany and Centers for Disease Control and Prevention, Atlanta, GA.)

potential adverse side effects, shunned the vaccine. Since then, the only persons actively being vaccinated are military service members in preparation for deployment and laboratory scientists working in LRN reference and national laboratories.

Diagnosis of smallpox

Detection and identification of smallpox virus are based on the patient's signs and symptoms, as defined by the CDC's acute, generalized vesicular or pustular rash protocol. Following this algorithm, patients seen by health care providers can be classified as being at low, moderate, or high risk for smallpox. Specimens collected from high-risk patients are transported to the CDC or another national laboratory for variola virus identification. Any individual suspected to have smallpox should be placed in isolation, typically in a negative-pressure room, to protect others against potential aerosol exposure.

Yersinia pestis

Throughout history, plague pandemics have demonstrated the efficacy of *Y. pestis* as an effective human pathogen. Natural outbreaks of plague are subject to international health regulations by notification of the WHO. The first recorded pandemic of plague ("Plague of Justinian") began in Egypt in A.D. 541 and lasted almost 200 years, decimating the human population by as much as 60% throughout North Africa, Europe, and parts of Asia. It is suggested that this event helped usher in the Dark Ages of Europe.

The second plague pandemic started in Asia in 1330 and spread into Europe in 1347. The spread of plague into and throughout Europe may have been facilitated by the Siege of Caffa in 1346. During the next 7 years, the plague killed as many as 20 million to 30 million Europeans, with perhaps another 20 million victims over the next 50 years. Localized outbreaks and smaller epidemics occurred up to the 17th century.

The third pandemic, the Hong Kong plague, began in the mid-19th century in China and lasted approximately 100 years. During the third pandemic, the plague gained access to the United States via ports in San Francisco in the early 1900s.

In 1894, Alexandre Yersin was the first person to describe the plague bacillus and fulfill Koch's postulates linking this organism with **bubonic plague**. The major vector for the plague bacillus is the rat flea, *Xenopsylla cheopis* (see Fig. 30.1), although many other fleas can transmit *Y. pestis*. More than 200 mammals can be involved in the transmission of plague. *Rattus rattus*, the black rat, is the rodent most attributed to urban outbreaks of plague. In the early years of the 20th century, large metropolitan areas along the West Coast of the United States experienced several outbreaks of plague until efforts were made to control or eradicate the rodents.

In the United States, sylvatic plague still occurs in the Southwest desert area, affecting mainly individuals who encounter reservoir hosts through occupational hazards, hunting, or camping. These reservoirs include deer mice, ground squirrels, prairie dogs, and other mammals. The most common vector in the United States is the flea *Diamanus montanus*.

Clinical manifestations

Y. pestis infection occurs in three distinct clinical forms: bubonic plague, septicemic plague, and pneumonic plague. Infections occur as a result of being bitten by an infected flea, handling contaminated materials, or inhaling aerosolized plague bacteria. Natural infections rarely occur in the United States. From 2000 to 2019, 96 cases of plague were reported in the United States, most of which occurred in the western part of the country, from New Mexico and Arizona north to Montana and Wyoming. New Mexico accounted for almost 50% of the reported cases.

Bubonic plague, the most common form of disease, results from the bite of a flea or by direct inoculation of an open skin wound by plague-infected material. As the organisms begin



Fig. 30.19 This patient has an axillary bubo and edema as a result of bubonic plague. (Courtesy Margaret Parsons and Dr. Karl F. Meyer and Centers for Disease Control and Prevention, Atlanta, GA.)



Fig. 30.20 The patient presented with symptoms of septicemic plague that included gangrene of the right hand, with necrosis of the fingers. (Courtesy Dr. Jack Poland and Centers for Disease Control and Prevention, Atlanta, GA.)

to proliferate at the inoculation site, a vesicle or ulcer forms. The plague bacteria are transported via the lymphatic system to the regional lymph nodes, usually the inguinal, axillary, or cervical lymph nodes. As the bacteria multiply, the affected lymph node becomes inflamed and extremely painful, forming the characteristic lymphadenopathy, or bubo, with evident edema (Fig. 30.19). **Buboes**, lymph nodes filled with inflammatory cells that can ulcerate, may be several centimeters in diameter. The location of the developing bubo is a strong indicator of the initial site of infection. Inguinal buboes suggest an infection on the lower extremities, whereas axillary buboes normally result from inoculation on the upper extremities. Although the bubo is a hallmark feature of bubonic plague, patients also experience rapid onset of fever, chills, headache, and general malaise.

In some patients, the lymph nodes become damaged, allowing hematogenous dissemination of the organism, causing secondary septicemic plague. Patients may also develop a primary form of septicemic plague in which no buboes are evident following infection, so the health care provider might not even suspect plague. Patients with the septicemic form of plague have symptoms like those of bubonic plague, but then progressively worsen as bacterial endotoxins trigger an immunologic cascade of events, ultimately leading to multiple organ failure, respiratory distress, and DIC, noted by petechiae and gangrene in the extremities (fingers, toes, and nose; Fig. 30.20). As the bacilli affect other organ systems, especially the lungs, brain, liver, and spleen, the patient can develop a secondary pneumonic plague, with dyspnea and hemoptysis, meningitis, and hepatic and splenic abscesses.

Individuals who develop the pneumonic form of plague are extremely infectious. They should be isolated, and respiratory precautions should be followed to reduce the spread of disease. Health care personnel should ensure that all individuals with close patient contact (within 2 m) wear a mask to avoid inhaling aerosolized plague bacteria and becoming infected. Because *Yersinia* is a nonsporulating, gram-negative organism, it cannot be dried and disseminated as easily as *B. anthracis*; it requires close contact and inhalation of moist contaminated droplets from a point source. If *Y. pestis* were to be weaponized, its optimal release would be via aerosol, and most patients would develop primary pneumonic plague.

This form is easily transmissible from person to person under conditions of close contact, continues the spread of disease, and increases overall mortality rates.

Specimen collection and preparation

Like anthrax, plague can manifest itself in numerous ways. If the patient has respiratory symptoms, lower respiratory tract secretions, such as sputum, bronchial wash, and trans-tracheal aspirate are the specimens of choice. Patients who appear septic and have a fever should have blood drawn for cultures. It is recommended that if *Y. pestis* infection is suspected, one set of blood cultures be held at room temperature because this organism grows faster at 22° to 28°C than at 35°C.

The specimen of choice for patients with buboes would be aspirated lesion fluid from the bubo or a biopsy of the lesion. Early buboes generally have little or no obtainable fluid, so 1 to 2 mL of saline should be injected into the bubo initially and then withdrawn to improve recovery of the organism. Care must be taken to avoid aerosol generation to minimize the risk of transmission during specimen collection.

Direct examination and initial culture

Gram-stained preparations of respiratory secretions, aspirates, and tissues infected with *Y. pestis* will reveal plump, gram-negative rods, approximately 1 to 2 µm by 0.5 µm. They may appear as single cells or in short chains; longer chains can form in liquid media. This organism often shows bipolar staining characteristics; that is, the ends are darker than the middle of the cell, resulting in what is described as a “safety pin” appearance. This staining characteristic is not specific for *Y. pestis* (*Pasteurella* and *Burkholderia* spp. can also display this type of morphology), and the Gram stain may not always be the best choice of stain to reveal the bipolar nature. Smears stained with Wayson or Wright stain may show more of the bipolar staining trait (Fig. 30.21).

Colonies of *Y. pestis* on SBA plates and other solid media develop slowly, requiring incubation for up to 48 to 72 hours for visible colony growth. Colonies are nonhemolytic and can have flattened edges, with a raised center demonstrating a

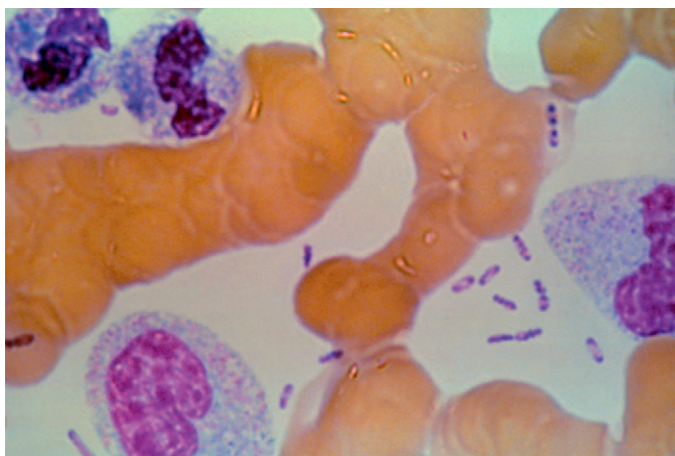


Fig. 30.21 Wright stain of *Yersinia pestis* from blood of a plague victim. Note the darker stained bipolar ends of the organism, resulting in the classic safety pin appearance. (Giemsa stain, $\times 1000$). (Courtesy Centers for Disease Control and Prevention, Atlanta, GA.)

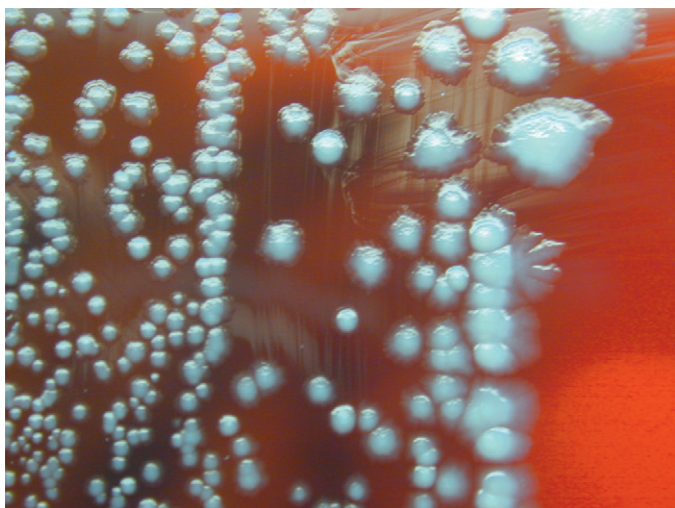


Fig. 30.22 *Yersinia pestis* on sheep blood agar, 72 hours of incubation. Colonies are gray-white to slightly yellow opaque, raised, with a irregular "fried egg" morphology; alternatively, colonies may have a "hammered copper" shiny surface. (Courtesy Larry Stauffer, Oregon State Public Health Laboratory, Hillsboro, OR, and Centers for Disease Control and Prevention, Atlanta, GA.)

"fried egg" appearance (Fig. 30.22). Older colonies are adherent to the agar, forming sticky, stringy strands when one tries to remove colonies from the plate (Fig. 30.23). Patient specimens inoculated into protein-based or thioglycollate broths may appear as long streamers, connected to the tube and growing into the broth. As these formations break, cottonlike fragments accumulate at the bottom of the tube, so-called *puffballs*.

Tests for presumptive identification

LRN sentinel protocols define a suspected *Y. pestis* isolate as a bipolar staining, nonmotile, non-spore-forming, gram-negative rod producing very small colonies after 24 hours of incubation at 35°C, whereas the colonies may appear slightly larger on plates incubated at room temperature. The organism will grow as small, nonlactose-fermenting colonies on MAC agar. *Y. pestis* is positive for catalase but negative for

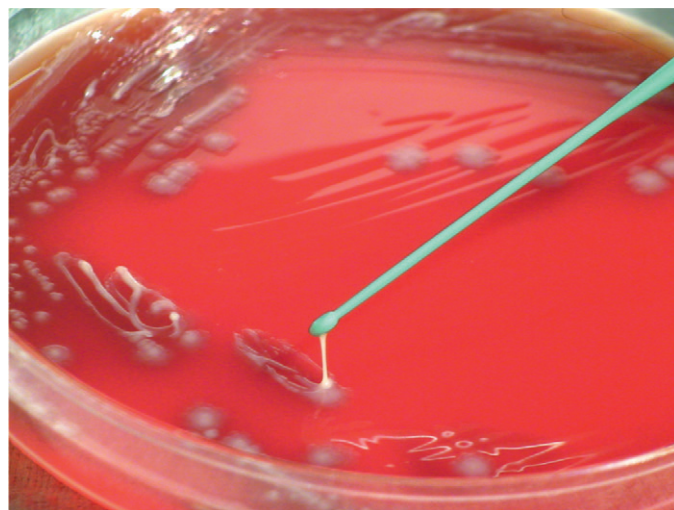


Fig. 30.23 Tenacity of *Yersinia pestis* colonies on sheep blood agar, 48 hours of incubation. Colonies are difficult to remove from plate and appear stringlike and sticky. (Courtesy Amanda Moore, Dr. Todd Parker, and Audra Marsh and Centers for Disease Control and Prevention, Atlanta, GA.)

oxidase and urease. Bacteria isolated from blood, respiratory specimens, or lesion aspirates that have these culture characteristics should be referred immediately to an LRN reference laboratory for definitive identification.

Other biological agents and toxins

Several other bacteria, viruses, fungi, and toxins are also select agents (see Table 30.1). Many of these are highly virulent or cause debilitating disease, even death, or they are less virulent but can be produced in large quantities. Importantly, they do not meet the criteria to be placed into tier 1. Nevertheless, their impact as biothreat agents should not be underestimated. *Salmonella*, *Shigella*, and *Escherichia* species are substantial food safety threats. Similarly, *Vibrio cholera*, *Cryptosporidium parvum*, and various plant and microbial toxins can be dangerous and costly water safety threats. Although large-scale delivery of many hemorrhagic fever viruses and arboviruses is less likely, their release into the general population would certainly instill fear and panic. Furthermore, the ongoing induction of antimicrobial resistance suggests that even organisms not categorized as select agents can become pathogens of increasing concern.

Pandemic preparedness and bioterrorism readiness

The terrorist attacks of September 11, 2001, and the mailing of anthrax spores in October 2001 changed the way of life for all people worldwide, especially those in the United States. The 9/11 Commission Report referred to it as "a day of unprecedented shock and suffering," noting that "the nation was unprepared." Reflections offered at the 15th memorial anniversary of 9/11 rightly identified the numerous changes made in disaster planning and emergency preparedness

since 2001. Plans at the national, state, and local levels have been developed to address mass casualties, displaced individuals, long-term health issues, and loss or disruption of government response. Incident command systems have been established, written protocols have been developed, and interoperability exercises, drills, and cross-training have been performed to respond more effectively, using an “all hazards” preparedness model. The 2009 H1N1 influenza pandemic, the 2015 Texas Ebola cases, and the 2019 COVID-19 pandemic were good tests of U.S. preparedness. Fortunately, the highly communicable H1N1 virus was not highly virulent, and the imported cases of Ebola, which caused infection in two health care workers, did not escape containment. However, COVID-19 challenged the very core of diagnostic testing and clinical response. The unfortunate loss of frontline health care providers, nurses, and laboratorians resulting from SARS-CoV-2 suggests that the United States is far from ready for bioterrorism, let alone a global pandemic that was anticipated months before arriving in the United States. These events provided many valuable lessons.

Concluding thoughts

Regardless of the type of threat agent, release of a select agent into the general population will certainly challenge the best sentinel laboratory. Biosafety practices aside, the lack of familiarity with rare organisms to have first-hand experience with culturing, biochemical testing, and sensitivity results and techniques could result in delayed recognition of the potential risk. Additionally, a bioterrorism event would suggest large numbers of people seeking diagnosis and care, thus producing a large influx of patient specimens for analysis. This would certainly require significant resources in terms of laboratory media, reagents, primers, probes, technical and clerical time, as well as security. Sound biorisk management practices should be part of any laboratory response plan.

Furthermore, it must be remembered that a bioterrorism event is a crime. Patient specimens are evidence in that crime and as such must be cataloged, processed, and protected in case the evidence is used in a court of law. Another point is the psychological stress placed by a bioterrorist event on the general population and health care professionals. The medical laboratory scientist is already on the frontline of our battles in health care. Adding the additional stress of working with potential select agents introduces another dimension for which the laboratory scientist should be prepared.

Forensic microbiology

A newer field of study combines forensic science and microbiology. The area of forensic microbiology involves determining the cause of death and identifying those individuals who have committed crimes. Biocrimes and bioterror fall under the umbrella of forensic microbiology. This field of study includes the clinical microbiologists who identify infectious agents, public health officials, and law enforcement agents. In forensic microbiology, it is not sufficient to identify the genus and species of a bacterial agent; the microbial strain

or signature must also be determined. This is important to track the source of the agent in an outbreak. Microbial signatures are generally determined based on nucleic acid characteristics, sometimes termed *DNA fingerprinting*. The methods used include PCR assays, gene sequencing, and pulsed-field gel electrophoresis (see [Chapter 11](#)).

Recently, determination of the stable isotope ratio of atoms, such as carbon, nitrogen, oxygen, and hydrogen, has been used to determine microbial signatures. The media used to cultivate bacteria contain isotopes that will be incorporated into the bacteria as they grow. The isotope pattern might be unique to a geographic location, allowing scientists to not only determine if the bacteria came from the same source but also identify the location where they were grown.

Another role of forensic microbiology is determining the cause of death in sudden infant death syndrome (SIDS), defined as sudden unexpected death in presumably healthy infants younger than 1 year of age. SIDS is the leading cause of death in neonates in developed countries and is a multifactorial condition involving sleeping position, genetic and immunologic factors, and infections. An estimated 10% of SIDS cases are caused by undiagnosed infections. Several infectious agents, including Epstein-Barr virus, cytomegalovirus, respiratory syncytial virus, *Neisseria meningitidis*, *Haemophilus influenzae*, and *Streptococcus pneumoniae*, have been linked to this syndrome. Determining an infectious cause is not always easy. Merely the detection or isolation of an infectious agent in postmortem tissue does not mean that the agent had a role in the death. It is possible that the bacteria simply colonized the patient before death and spread after death. Forensic microbiology can also play a role in ruling out shaken baby syndrome, which is characterized by unexplainable intracranial and retinal hemorrhages and acute encephalopathy. Staphylococcal septicemia and meningitis can produce similar clinical findings, and if detected postmortem, can exonerate individuals accused of homicide.

Forensic microbiology has been investigated as a way to help determine drowning as a cause of death. The diagnosis of drowning can be difficult and is often made by the exclusion of other causes. Forensic scientists have looked for microorganisms, such as diatoms, in the tissue of suspected drowning victims. Diatoms are single-celled organisms found in freshwater, saltwater, soil, air, and food. It is believed that their presence in postmortem tissue, such as the liver, bone marrow, and kidneys, proves the hematogenous spread of the organisms from the lungs from a beating heart before death. However, testing for diatoms is labor-intensive, and diatoms have been notably absent in cases of known drownings. Because of these drawbacks, forensic scientists have investigated the detection of fecal bacteria in postmortem tissue. Some bodies of water are contaminated with animal feces and contain bacteria colonizing the GI tract, such as *Escherichia coli* and *Enterococcus faecalis*. Testing the drowning medium as well as the postmortem tissue is required. If the same bacteria are found in the tissue as those in the drowning medium, it can be concluded that the likely cause of death was drowning. Recently, scientists have investigated using aquatic bacteria (bacterioplankton) in suspected drowning cases. These agents are more abundant and much smaller than diatoms, making it easier for them to enter the bloodstream and reach the viscera.

Aeromonas, *Vibrio*, and *Photobacterium* are the genera being studied. The bacterial genome can be detected using PCR or next-generation sequencing.

Determining time of death, or postmortem interval (PMI), is often critical information in an investigation into a suspicious death. PMI is classically determined based on changes in the body after death (e.g., body temperature, decomposition, rigor mortis, insect infestation) combined with information gathered from investigations by law enforcement agents. Recently, scientists have been investigating changes in the microbiome that occur after death to estimate the PMI. The postmortem microbiome can be divided into two components: the thanatomicrobiome (*thanatos*, Greek for “death”) and the epinecrotic microbiome (on the surface of decomposing remains). The thanatomicrobiome is composed of mainly bacteria and fungi present in the blood and internal organs, while the epinecrotic microbiome is composed of the microorganisms residing in or on the surface (e.g., superficial epithelial tissues, oral mucosal membranes). After death, because of the cessation of innate and adaptive immune responses, gut bacteria begin to spread to the surrounding tissues and organs and move along the circulatory and lymphatic systems, initiating the decomposition process. Typically, as decomposition progresses, microbial community diversity decreases, resulting in only a few genera predominating. There is a gradual shift from aerobic-based communities to communities based primarily on anaerobes. As more data are collected, it might be possible to develop a microbiome “calendar” with several time points that can match the bacteria detected from a cadaver to a specific time point.

POINTS TO REMEMBER

- Bioterrorism is the use (or threatened use) of biological agents to harm humans, animals, or crops to cause civil unrest. Biological warfare is the use of biological weapons to gain a military advantage.
- Many of the biological agents, both bacteria and viruses, used in bioterror are zoonotic.
- Many biological agents used in bioterror can also be isolated from patients with naturally occurring infections.
- Many infections caused by bioterror agents produce similar nonspecific symptoms in patients; therefore the laboratory scientist plays a key role in the diagnosis.
- Sentinel laboratories play a crucial role in the rapid detection and reporting of potential biological agents. As such, the laboratory scientist must be aware of the select agent rules, proper biosafety containment, and biosecurity and perform a biological risk assessment.
- The ASM provides information on minimal tests for the identification of potential bioterror agents for sentinel laboratories to use.
- Clinical microbiologists in sentinel laboratories should immediately notify their state public health laboratories when a potential biological agent cannot be ruled out and receive instructions and guidance on the chain of custody and shipping.
- The field of forensic microbiology includes the investigation of bioterror, biocrimes, unexpected deaths, and sometimes drownings.

LEARNING ASSESSMENT QUESTIONS

1. Which statement is correct regarding bioterrorism?
 - a. It only involves foodborne transmission.
 - b. It is the accidental release of viruses, bacteria, or other germs used to cause illness or death in people, animals, or plants.
 - c. It is the intentional release of viruses, bacteria, or other agents used to cause illness or death in people, animals, or plants to cause panic.
 - d. It is the release of viruses, bacteria, or other agents by the government to cause illness or death in people, animals, or plants.
2. Which of the following is (are) used to select an agent for bioterrorism?
 - a. Lethality
 - b. Ease of delivery
 - c. Ease of acquisition
 - d. All of the above
3. You might suspect the release of a bioterrorism agent if which of the following occurs?
 - a. Large numbers of people seek treatment at hospital emergency departments.
 - b. There are reports of unusual microorganisms being isolated from sick people.
 - c. There is a large die-off of animals in your area.
 - d. All of the above.
4. How are bioterrorism agents classified? (*Select all that apply.*)
 - a. UN 3373
 - b. Tier 1
 - c. Class 6.1
 - d. Category Y
5. Which of the following methods is used to identify agents of bioterrorism?
 - a. Culture
 - b. Biochemical analysis
 - c. Genetic sequencing
 - d. All of the above
6. Which of the following diseases is transmitted principally by fleas?
 - a. Plague
 - b. Tularemia
 - c. Anthrax
 - d. Rocky Mountain spotted fever
7. Which organism produces a toxin used as a bioterrorism agent?
 - a. *F. tularensis*
 - b. *Y. pestis*
 - c. *C. botulinum*
 - d. *B. anthracis*
8. Depending on the agent, all incidents involving select agents should be reported to local and state public health authorities as well as the:
 - a. Central Intelligence Agency
 - b. U.S. Department of Homeland Security
 - c. U.S. Centers for Disease Control and Prevention and the U.S. Department of Agriculture
 - d. U.S. Department of Justice and the U.S. Postal Service
9. Biosafety level designation would change from _____ to _____ when moving from diagnosing tuberculosis in specimens using direct staining of smears to culturing mycobacteria.
 - a. BSL-2 to BSL-3
 - b. BSL-3 to BSL-4

- c. BSL-2 to BSL-4
- d. It would not change.
10. The biosafety level of a laboratory is defined by which of the following?
 - a. Risk group and procedures of the agent used in the laboratory
 - b. Biochemical virulence factors of the agent used in the laboratory
 - c. Risk group and risk assessment of the agent used in the laboratory
 - d. Gram stain and microscopy of the most virulent agent used in the laboratory
11. Shaken baby syndrome can be confused with which of the following?
 - a. Sudden infant death syndrome
 - b. Bacterial septicemia and meningitis
 - c. Variola virus infection
 - d. Tularemia
12. The finding of diatoms in a cadaver found in a body of water is suggestive of which of the following?
 - a. Stabbing
 - b. Encephalitis
 - c. Strangulation
 - d. Drowning
13. Many years ago, *Bacillus anthracis* and variola major virus were classified as BSL-2 agents. Those classifications have been modified to fall under higher BSLs. Why was a change necessary?
14. What important roles do reference laboratories perform within the LRN?
15. Which unique pathogenic features distinguish tier 1 agents from those in the non-tier 1 group?
16. For each of the following organisms, what specific identification characteristics (e.g., Gram stain morphology and biochemical test results) must be met before a sentinel laboratory must contact an LRN reference laboratory to submit a sample?
 - a. *Bacillus anthracis*
 - b. *Yersinia pestis*
 - c. *Francisella tularensis*
 - d. *Burkholderia* spp.
17. What are the recommended clinical specimens to submit for patients potentially exposed to the following?
 - a. Ebola virus or Marburg virus
 - b. *Clostridium botulinum* toxin
 - c. Inhalational anthrax
 - d. Variola virus

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Biofilms: architects of disease

Donald C. Lehman

CHAPTER OUTLINE

Microbial biofilms defined, 777

Biofilms: community of cells, 778

Stages in biofilm formation, 778

Architecture of biofilms, 780

Biofilm properties, 780

Mechanisms of pathogenicity, 780

Role of biofilms in attachment, 781

Benefit of biofilms for metabolism, 781

Biofilms as a defense mechanism, 781

Gene transfer and expression, 782

Disaggregation, 782

Diseases associated with biofilms, 782

Dental biofilms, 782

Cystic fibrosis biofilm, 784

Chronic upper respiratory tract infections, 784

The changing paradigm of microbial infections, 784

Laboratory consequences associated with biofilms, 785

Detection of biofilms and in vitro formation, 786

Potential interventions, 786

Biofilm models for studying antimicrobials, 787

Bibliography, 789

OBJECTIVES

After reading and studying this chapter, you should be able to:

1. Describe the proposed origin of biofilms, in a historic perspective, as a means of microbial survival.
2. Define *biofilm*.
3. Compare the planktonic phenotype with the sessile phenotype.
4. Describe the interaction of multiple cells in a community.
5. Describe the five stages of biofilm formation.
6. Discuss the architecture of biofilms.

7. Describe the potential virulence mechanisms of biofilms.
8. Discuss the role of biofilm formation in periodontal disease.
9. Explain the role of indwelling medical devices and the pathogenesis associated with biofilms.
10. Discuss the consequence of biofilms for the clinical laboratory, particularly false-negative cultures, viable but nonculturable bacteria, low colony counts, inappropriate specimen collection, and loss of susceptibility to antimicrobial agents.
11. Evaluate the various biofilm interventions and their limitations.
12. Analyze laboratory methods that may be used to study biofilms.
13. Describe how bacterial cells communicate.

KEY TERMS

Biofilm

Biofouling

Exopolysaccharide (EPS)

Persister cell

Pheromone

Planktonic

Quorum sensing (QS)

Quorum-sensing inhibitor (QSI)

Sessile

Case in point

An 83-year-old female patient was admitted to the hospital after developing a fever and having a hypotensive episode while in residence at a nursing home. The patient had a clinical history of rheumatoid arthritis, hypertension, atrioventricular block, gastroesophageal reflux disease, deep vein thrombosis with pulmonary embolism, and depression. The patient also had a history of recurrent prosthetic left knee joint infections after total knee arthroplasty 2 years earlier. A vancomycin-resistant enterococcus (VRE), *Enterococcus faecium*, was the predominant organism isolated in synovial fluid from her knee. The patient was given multiple antimicrobial regimens with numerous revisions. The course of her illness was complicated by pancytopenia, most likely caused by injury to the bone marrow, possibly from a drug reaction. At this admission, blood and synovial

fluid cultures were collected. The patient was given antimicrobial therapy and supportive care, but her condition worsened, and “comfort measures only” were instituted. When the patient died, an autopsy was requested by the family. The final pathologic diagnoses were as follows:

- Nonhealing chronic wound infection caused by VRE associated with prosthetic left knee joint and clinical history of sepsis
- Pulmonary edema and pleural and peritoneal effusions
- Pulmonary vascular calcifications and cardiomegaly

Issues to consider

After reading the patient's case history, consider:

- The role and significance of biofilms in infectious diseases
- The difficulty of successfully treating infections involving biofilms
- The consequences of untreated biofilm diseases
- Whether laboratory standard practices should be altered to meet the challenges presented by biofilm-associated infections

Biofilms are three-dimensional communities of microorganisms attached to a solid surface; the surface can be abiotic (nonliving) or living tissue. Organisms in a biofilm are specialized and have a great deal of genetic energy. Given the selective pressures of certain environments, notably aquatic systems, biofilms are the preferred method of growth. Their unique structure, which evolves over time, allows the development of a cohesive, robust community of cells, with interspecies communication driven by the principle of survival. Microorganisms, prokaryotic or eukaryotic, have the potential to live in one of two phenotypes: sessile or planktonic. The **sessile** phenotype results from attachment and usually develops into a multispecies biofilm that has unique characteristics, making it similar in many ways to hydrated polymers. **Planktonic** forms are free-floating microorganisms.

Biofilms can be simple monospecies or complex cobiofilms. Cobiofilms, or multispecies biofilms, predominate in most environmental sites (Fig. 31.1). Biofilms can form on a variety of surfaces, such as on ships' hulls and rocks in rivers and streams. Biofilms that occur inside pipes carrying water

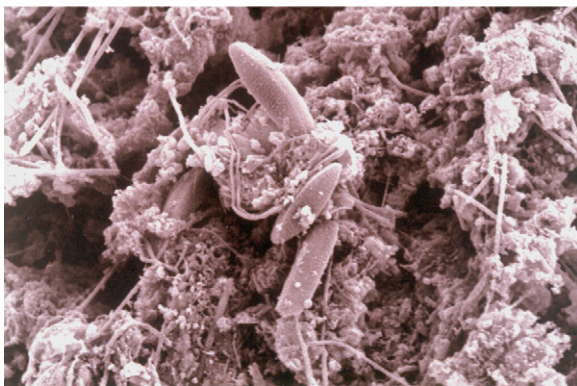


Fig. 31.1 Natural biofilm on a metal surface. This is a scanning electron micrograph of a native biofilm that developed on a mild steel surface in an 8-week period in an industrial water system ($\times 20,000$). (Courtesy Rodney Donlan and Donald Gibbon, from the American Society for Microbiology MicrobeLibrary.)

for the manufacture of drugs make it unsafe because of the presence of the organisms and toxins such as endotoxins that can induce septic shock in individuals receiving the drugs. Biofilms inside pipes that carry water can make the water not potable. The process of biofilms forming on surfaces of pipes carrying potable water is referred to as **biofouling**.

Cobiofilms of bacteria and fungi have been found in ventilator systems of airplanes, ice machines in restaurants and bars, and mines, in which unique configurations have been found that result from the leeching process, creating stress on the inanimate surface. Biofilms that involve mastitis in farm animals affect agriculture, and biofilms in barrels can cause wine to spoil, affecting the wine industry. Cobiofilms of *Pseudomonas aeruginosa* and *Burkholderia cepacia* are associated with serious lung infections in patients with cystic fibrosis (CF). Monospecies biofilms are associated with a variety of infections. Medically important biofilms are produced by bacteria such as *P. aeruginosa*, *P. fluorescens*, *S. aureus*, and *S. epidermidis*.

It is important for clinical microbiologists to become familiar with the role biofilms play in human disease. As people live longer or are in a prolonged debilitated state of health and/or undergo invasive procedures, they are more prone to infections by opportunistic pathogens and the development of biofilms. Recently, the challenges that biofilms present to the clinical microbiology laboratory have been recognized. These include problems in performing susceptibility testing, interpreting the clinical significance of susceptibility test results, recovery of viable but nonculturable organisms in a mixed-species biofilm, and the recognition that biofilms transform from gram-positive to gram-negative in an environment that is difficult to duplicate. This chapter presents an overview of biofilm properties and composition and discusses the consequences and challenges associated with biofilms that the medical laboratory scientist must confront. Diseases associated with biofilms are presented, and potential interventions are introduced.

Microbial biofilms defined

Several definitions exist for the term *biofilm*. These have evolved over time as our understanding of the forces and features of biofilms has progressed. However, the definition in Box 31.1 seems to endure and puts into perspective the concept of developmental biology and the uniqueness and cooperation required among organisms within the biofilm. One such definition recognizes the components and imperative ingredients that characterize this multiorganism cooperative population. According to the definition, microbially derived sessile communities are characterized by cells irreversibly attached to a

BOX 31.1 Biofilm definition

A biofilm is a primitive developmental biological system in which spatial organization of the cells within the matrix optimizes the use of available nutritional resources. An immobilized enzyme system is formed in which the milieu and enzyme activities are constantly changing and evolving to an appropriate steady state. The steady state can be radically altered by applying physical factors, such as high shear force.

substratum, or interfaced to each other, embedded in a matrix of an extracellular polymeric substance that produce and exhibit an altered phenotype with respect to growth rate and gene transcription.

Microbial biofilms (biofilm phenotype) are as common as planktonic microorganisms (planktonic phenotype). Almost any liquid environment in which organisms are subject to shear forces and nutrition alteration selects for upregulation—that is, increased expression of genes consistent with the biofilm phenotype. Planktonic phenotype and biofilm phenotype cells can have the same genetic potential but express a different phenotype. Given the appropriate environmental selective pressures, a significant number of bacteria will preferentially develop a biofilm phenotype. It is important to recognize that the two phenotypes are not mutually exclusive, but that the ratio of the planktonic phenotype to the biofilm phenotype can be a predictor of associated disease. Biofilms are a universal mechanism for survival, horizontal gene transfer, and growth as well as a probable ancestor of the planktonic free-floating microorganisms. The planktonic phenotype is genetically expensive, with sophisticated chemotactic and mobility mechanisms that may have developed after the biofilm phenotype. The primary purpose of the planktonic phenotype is dissemination and colonization of new habitats.

There is growing evidence that the biofilm was the origin of survival and necessary for communication among microbes. Approximately 3.4 billion years ago, biofilms allowed microbes to survive, grow, and adapt in a hostile environment, including extreme pH and temperature and lack of water. As the Earth cooled and moved toward a multispecies adaptation with less environmental pressure, organisms were able to develop the ability to transfer to different ecologic niches and live outside communities. Hence, the postbiofilm planktonic phenotype evolved, which had a reduced need for communities for survival.

Biofilms: community of cells

Although certain constituents are common to all biofilms, the contribution of the host relative to the microorganisms,

such as immunologic components and the physical location, has an antibody influence on biofilm structure. Several key environmental and cultural characteristics affect the selection of multispecies biofilm inhabitants (Fig. 31.2). These features include (1) species attachment efficiency, (2) genotypic factors, (3) cyclic stage of biofilm, (4) substrata, (5) nutritional resources, (6) mechanical factors and shear forces, (7) physicochemical environment, and (8) anti-infective hostile pressure forces. Of these, the four most important are organism attachment efficiency, nutritional resources, substrata, and environmental shear stress or force. Shear stress, probably the most important characteristic, affects the physical morphology and dynamic behavior of the biofilm. The steady-state kinetics of the organisms within the biofilm can be radically altered by physical factors such as high shear, and the shear rate will determine the rate of erosion of cells and of the matrix from the biofilm.

As a biofilm forms, streamers of cells extend from the surface. These cells can break off and establish new biofilms downstream. Not all microorganisms change with a high efficiency of upregulation between the planktonic and biofilm phenotypes. It is no coincidence that coagulase-negative staphylococci, *P. aeruginosa*, and *Candida albicans* are some of the most efficient microbes prone to upregulation when selective pressures magnify the need for survival in a biofilm community. These microorganisms are also important human pathogens. Both prokaryotes and eukaryotes are important in biofilm-associated diseases. In particular, a cobiofilm of *Candida* and pseudomonad species is very difficult to eliminate because of its drug resistance. A cobiofilm increases in robustness and stability with maturity and the number of organisms within the community.

Stages in biofilm formation

Biofilm formation progresses through well-regulated steps that include attachment of cells to a substrate, growth and aggregation of cells into microcolonies, and maturation and maintenance of architecture. During the process, many

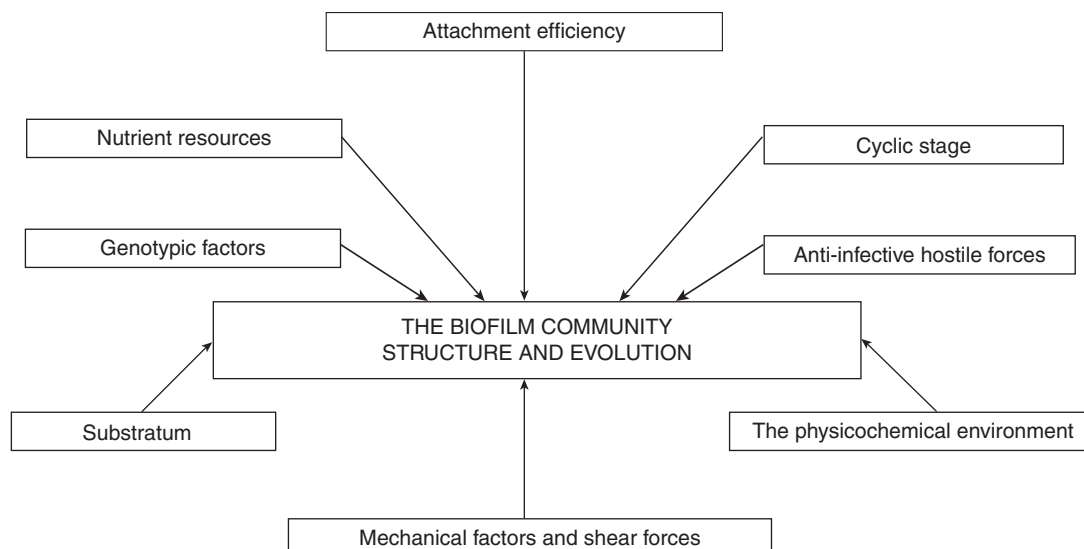


Fig. 31.2 Environmental and cultural factors that affect biofilm development.

species of microorganisms progress through multiple developmental stages. There is a five-stage universal growth cycle of a biofilm with common characteristics independent of the phenotype of the organism.

Stage I is the attachment phase, which can take only seconds to start and is likely induced by environmental signals. These signals differ by organism but include changes in nutrients and nutrient concentrations; pH, temperature, oxygen, and iron concentration; and osmolality. Even though biofilms can form on any surface, rough surfaces are more susceptible to biofilm formation. This is likely because of the reduction of shear forces and increased surface area. Biofilms also tend to form more readily on hydrophobic material, such as Teflon and other plastics (polymers), than on glass and metals. [Table 31.1](#) lists some key factors in biofilm formation. The initial binding in stage I is reversible as some cells detach from the substratum. Binding to a surface is mediated by several adhesion molecules and pili. One example is polysaccharide intercellular adhesion, which is produced by enzymes encoded by the gene locus *ica* found in *S. aureus* and *S. epidermidis*. In *Escherichia coli*, there are genes that encode a similar polymer associated with adhesion, designated *pga*, and they share sequence similarity with the staphylococcal genes *ica*. It has been observed that exposure to subinhibitory concentrations of various antimicrobial agents activate the expression of *ica*.

After attachment, the cells undergo changes in gene expression that lead to existence on a solid surface. It has been shown with *C. albicans* that switching from yeast forms to hyphal growth is important in biofilm development. During stage I, bacterial cells exhibit a logarithmic growth rate. Stage II is characterized as irreversible binding and begins minutes after stage I. During stage II, cell aggregates are formed, and motility is decreased. Toward the end of this stage, **exopolysaccharide** (EPS) is produced, which can trap nutrients and planktonic bacteria. In staphylococci and enterococci, iron deprivation and osmotic stress induce the expression of genes encoding proteins that synthesize EPS. It has been shown that alginate, an EPS, is increased threefold to fivefold in *P. aeruginosa* after attachment to a solid surface compared with planktonic cells.

When cell aggregates become progressively layered, with a thickness greater than 10 μm , the biofilm is in stage III. This stage is also referred to as *maturation 1*. When biofilms reach their ultimate thickness, generally greater than 100 μm , this is referred to as stage IV or *maturation 2*. It takes a biofilm several days to reach this stage. Some of the cell aggregates are displaced from the substratum but remain trapped in the biofilm EPS. During stage V, cell dispersion is noted. Some of the bacteria develop the planktonic phenotype and leave the biofilm. Stage V begins several days after stage IV.

Cycling of stage I to stage V is based on the organism pool and timing of the growth characterization of the individual members. The formation of a biofilm has been compared with cell differentiation of a multicellular organism. One reason for this comparison is that microorganisms in a biofilm express great variability in metabolic activities. A biofilm is a dynamic system, containing many species of bacteria with different characteristics. It is also recognized that there is a constant assessment of the need to remain within the cycle, attached or free-floating, and, again, neither is mutually exclusive. Certain organisms, however, have evolved a much more efficient means of maintaining one phenotype or the other.

The organization of biofilms may take several forms, but it is important to recognize that a nonuniform spatial pattern and nonuniform susceptibility with physiologic heterogeneity equals survival in a harsh environment, such as one containing antimicrobial agents. **Persister cells**, essentially metabolically inert microorganisms analogous to bacterial spores, are present in all biofilms and bacterial populations. Persister cells are hypothesized to have disabled programmed cell death, or apoptosis. These cells have the greatest potential for maintenance of the gene pool and resistance to environmental stress, including antimicrobial agents.

Much of the development and structural integrity of the biofilm depends on **quorum sensing** (QS) by QS autoinducers. QS is the ability to use extracellular molecules, **pheromones**, for enhanced communication among bacteria not only of the same genera and species but also among bacteria of different genera and species. The ability of bacteria to communicate among themselves shows a behavior analogous to what occurs among animals and people. The most striking element of QS in gram-negative organisms is the highly conserved set of three-component regulatory networks used by these organisms to efficiently regulate a wide variety of bacterial density-dependent activities, such as metabolism, virulence, chemotaxis, and notably, biofilm production and maturation.

The viability of the biofilm community depends on stress response genes and cell signaling from the cells via QS through biochemicals. QS is essential for biofilm formation, triggering expression of some genes and downregulation (silencing or reducing expression) of others. Pheromones are different for gram-positive and gram-negative bacteria. Gram-negative bacteria use low-molecular-weight homoserine molecules, such as *N*-acyl homoserine lactone, whereas gram-positive bacteria use oligopeptides or proteins. The role of QS for different organisms mechanistically in a biofilm community remains to be understood. Physical contact, or cell aggregation, can also provide signals that are important in biofilm formation. Challenges in the study of biofilms include:

1. Which parameters of a biofilm community influence the onset of QS and subsequent patterns of gene expression?
2. What are the functional challenges of QS?
3. Does induction of QS influence the pathogenic potential of biofilm communities?
4. Does induction of QS alter the antimicrobial tolerance of a biofilm community?
5. Are there organisms in a biofilm community that eliminate or reduce signal transfer among organisms?
6. Does interspecies signaling occur in mixed-species systems, and what triggers this signaling?

Table 31.1 Important variables in cell attachment and biofilm formation

Properties of substratum	Properties of surrounding fluid	Properties of the cell
Texture or roughness	Flow velocity	Cell surface
Hydrophobicity	pH	hydrophobicity
Conditioning film	Temperature	Fimbriae
	Cations	Flagella
	Available nutrients	Adhesion molecules

Not only does QS occur among bacteria, but it has now become evident that host tissue can exploit bacterial QS to influence commensal bacteria. In addition, research suggests that serotonin, a neurotransmitter responsible for mood in the brain and motility in the gut, can also act as a bacterial signaling molecule for pathogenic bacteria. In an animal model, it was shown that serotonin enhanced the virulence of *P. aeruginosa*.

Architecture of biofilms

It is important to recognize the interplay of biofilm and planktonic phenotypes and the three-dimensional architecture of the biofilm as being universal, whether the biofilms are in animals, agricultural environments, or industrial settings. Biofilms are composed of stalks of mushroom-shaped microcolonies attached to the substratum and surrounded by EPS. The biofilm matrix contains EPS, proteins, and DNA; EPS constitutes 50% to 90% of the organic carbon in the matrix. Biofilms are hydrated, with fluid-filled channels running throughout. The fluid-filled channels facilitate the exchange of nutrients and carry away waste products. In addition, motile microorganisms can be found in the aqueous portion of a biofilm. [Table 31.2](#) lists some commonly found constituents of biofilms.

The three-dimensional architecture of the mature biofilm often has three layers. The outermost layer is exposed to the highest concentration of nutrients and oxygen. It contains the most active organisms, which primarily resemble the structure and activity of their planktonic counterparts. These organisms are generally closely aligned, with selective pressures. Even though they are also part of the EPS, the organisms can slough off and initiate biofilm formation downstream. The second layer of the biofilm architecture is an intermediate level. Organisms in this layer downregulate their metabolic activity somewhat, but they clearly have the capacity to use nutrients and exchange genes and have the potential for multiple drug resistance. This spatial arrangement, physiologic heterogeneity, and nonuniformity are based on the proximity of microorganisms aligning next to one another. They benefit from that alignment, and it is not by chance. The innermost layer of cells is attached to the substratum and represents the earliest part of the biofilm. These microorganisms are the least metabolically active, and this is where most persister cells are located. Generally, the innermost layer represents the inheritance for future populations that transfer genes laterally.

Table 31.2 Components of biofilms	
Component	Percentage of matrix
Water	≤97%
Microbial cells	2%–5%; many species
Exopolysaccharide	1%–2%
Proteins	<1%–2%
DNA and RNA	<1%–2%

Biofilm properties

Biofilm phenotypes cannot be defined by traditional principles of microbiology. Cell behavior in response to stress is much more than a group of microbes attached to a surface. In fact, the properties of a biofilm resemble the properties of organic polymers. Two key properties of a biofilm are attributable to their hydrated polymer gel-like material. First, biofilms exhibit viscoelasticity (i.e., viscous and elastic properties). The consequence of this viscoelastic property is magnified in the time-dependent response of biofilms. In short periods, biofilms absorb increased shear by behaving elastically. In contrast, for long periods, shear is dissipated through viscous flow by a streamlining of the structure to reduce drag.

This viscoelastic property can be measured by using principles of rheology, meaning that everything flows. The Greek philosopher Heraclitus (540–480 BC) of Ephesus said: “Everything flows and nothing abides; everything gives way and nothing stays fixed.” In a more practical sense, will it flow, or will it fracture? The outcome is determined by the applied forces. This also explains the phenomenon of creeping, or rolling, that allows a multispecies biofilm to move like lava flow when attached to an inanimate or abiotic surface. There is no detachment; rather, it is the movement of a protective community down a surface, as defined by viscoelastic properties of a material-like substance. This is of major concern when addressing, for example, movements of biofilms within the lumen of an endotracheal tube or a central catheter line.

Second, not only do biofilms share properties of organic polymers, but they also have many properties common to a neoplasia, another cell community. A neoplasia is a eukaryotic irreversible genotype. In contrast, members of a biofilm have reversible phenotypes, and they are more often prokaryotes. However, given these differences, several features are shared by a neoplasia and a biofilm to some degree. Common to both are cycling or staging, metastasis, loss of contact inhibition, and cell-to-cell signaling. Similarities can also be observed in the three-dimensional architecture, heterogeneity, and virulence phenotype. The sharing of characteristics between a neoplasia and a biofilm highlights the fact that biofilms are different from their free-floating counterparts.

Mechanisms of pathogenicity

A great deal has been learned about the structure, function, and properties of biofilms. The mechanisms of pathogenicity, on the other hand, are a different matter.

- Do biofilms contribute to infectious disease?
- If so, what are the mechanisms of pathogenicity?
- Are some biofilms more pathogenic than others?

Some observations appear to provide evidence of the significance of biofilms in the mechanisms of disease. [Box 31.2](#) lists several potential pathogenic mechanisms concerning biofilms and disease. In the environment and in patients, biofilms are the most advantageous method of growth for most microorganisms. Biofilms originate because of selective

BOX 31.2 Biofilm pathogenicity and disease

- Allows attachment to a solid surface
- "Division of labor" increases metabolic efficiency of the community
- Evades host defenses such as phagocytosis and antibodies
- Produces a high density of microorganisms
- Horizontal gene transfer can result in more virulent strains of microorganisms
- Produces a large concentration of toxins
- Protects against antimicrobial agents
- Can aid penetration of host tissue
- Detachment of microbial aggregates transmits microorganisms to other body sites

pressures in an environment in which survival can be difficult, such as the presence of host defenses and antimicrobial agents. Transitioning from acute to chronic infections is frequently associated with biofilm formation.

Case check 31.1

An aggregation substance protein produced by *Enterococcus faecalis* stimulates the production of a biofilm. The protein induces aggregation formation between *E. faecalis* cells. This protein can also facilitate adherence to host tissue. The patient in the Case in Point had a chronic VRE infection associated with a prosthetic left knee joint. It is likely that biofilm formation was involved.

Role of biofilms in attachment

Colonization is often a critical first step in the development of an infectious disease, and biofilms may be an important mechanism of attachment to host tissue and indwelling medical devices (IMDs). Intravascular catheters, urinary catheters, pacemakers, heart valves, stents, and orthopedic implants are just a few examples of IMDs. Initial colonization involves bacterial adhesions such as surface proteins (e.g., teichoic acid, lipoteichoic acid) and pili. Motility is also important for initial colonization. However, after bacteria adhere to a surface and biofilm construction begins, changes in gene expression result in the suppression of flagella and increased expression of adhesion molecules. However, once a biofilm is established, adhesion mechanisms are no longer needed.

Benefit of biofilms for metabolism

Biofilms allow members of the microbial community to withstand the shear forces of blood and urine flow, presumably keeping the microorganisms in a nutrient-rich environment. It was demonstrated with certain organisms, such as the pseudomonads, *E. coli*, and *Vibrio cholerae*, that the presence of an abundant usable carbon source (e.g., glucose) stimulates the production of EPS. The EPS can trap more microorganisms and can serve as a glucose storage mechanism at times when carbohydrates are plentiful. As nutrients in the surrounding environment become scarce, gene expression changes, and some bacteria in the biofilm detach and become planktonic.

Because of the extreme variability in available nutrients, pH, and oxygen concentration in a large complex biofilm, there is great heterogeneity in the metabolic activity of microorganisms in the community. Even in monospecies biofilms, metabolic requirements and activities differ. This variability may result in a so-called *division of labor*, whereby the metabolic efficiency of the microbial population is increased. Sharing of metabolic by-products is the basis of a beneficial symbiotic relationship. It has been proposed that bacteria living in a biofilm can exhibit mutually beneficial behavior. Evidence indicates that bacteria can undergo programmed cell death. Death of some cells in the community could relieve the metabolic load by releasing nutrients for nearby cells. This would help the biofilm to survive. One of the major benefits of the biofilm configuration is its ability to produce many organisms per unit mass. Generally, in broth cultures, it is difficult to obtain more than 10^8 cells/mL, but with configuration and upregulation of a biofilm phenotype, microorganisms can reach concentrations of 10^{12} cells/mL or higher.

Biofilms as a defense mechanism

Biofilms can afford protection from host defenses, including oxygen-reactive molecules, antimicrobial proteins like antibodies, antimicrobial drugs, and phagocytosis. In addition, biofilms protect against pH changes and nutrient deprivation. A major benefit of the biofilm to microorganisms is its interference with immune function, particularly the activity of white blood cells. The biofilm provides a physical barrier that prevents phagocytic cells, such as polymorphonuclear (PMN) cells, from penetrating and phagocytizing the bacteria. When confronting a biofilm, PMN cells will degranulate and cause damage to nearby host tissue. In periodontal disease, the consequence of interrupted white blood cell function is the production of large quantities of collagenase of human origin that causes deterioration of support bone. In addition, biofilms can hide microbial antigens, inhibiting an immune response by preventing antibody formation, and even if antibodies are formed, they block interaction with bacterial antigens. Antibodies cannot diffuse through a biofilm, but immune complexes can form on the biofilm surface and nearby tissue, producing inflammation.

How a biofilm provides protection to its members is not completely known. Possibly, sticky EPS provides a physical barrier to penetration by phagocytic cells and antimicrobial molecules. Also, the heterogeneity of microbial metabolic activity may play a role. It is known that slow-growing organisms are more resistant to antimicrobial agents. This seems counterintuitive because one argument for forming a biofilm is the presence of a nutrient-rich environment; however, those microorganisms living near the substratum exist in a nutrient-poor environment and therefore grow slowly. The upregulation of the biofilm phenotype by microorganisms has the potential to outcompete normal biota. This is also particularly appropriate as bacteria transition from acute infections to chronic infections, more likely associated with the biofilm phenotype and the coaggregates of multispecies biofilms. The early bacterial population during an acute infection will be exceeded later in the course of the disease, when the organisms reach a biofilm configuration. The microbial

population is also often susceptible to antimicrobial agents early in the infections, as determined by genotyping, but becomes much more resistant as the population shifts to a biofilm phenotype. Removal of the selective pressure is critical in shifting the ratio of biofilm phenotype to planktonic phenotype back to the planktonic phenotype in the nondisease state.

Biofilms afford bacteria the opportunity to become tolerant to antimicrobial agents. Bacterial tolerance is defined as resistance to antimicrobial agents in bacteria residing in a biofilm as a result of existing genes. Susceptibility is restored when the bacteria leave the biofilm. It is critical to remember that the classic mechanisms of genetically encoded bacterial resistance can also operate in bacterial biofilms. Multidrug efflux pumps, antibiotic-modifying enzymes, and bacterial modifications are all viable modes of resistance for biofilms. However, these classic resistance mechanisms do not explain the resistance observed in bacteria that do not possess known antimicrobial-resistance genes.

Several hypotheses may explain biofilm resistance to antimicrobial agents: (1) inability of an antimicrobial compound to penetrate all areas of the biofilm; (2) altered areas of metabolic activity of the bacteria or reduced growth rate, rendering antimicrobial agents such as penicillin, which can inhibit the growth of only actively growing bacteria, ineffective; (3) persister cells, which are dormant, nondividing cells; and (4) differential gene expression within the biofilm community, which may allow certain bacteria to protect other bacteria by producing needed nutrients, compounds that inactivate antimicrobials (e.g., penicillinase), or other necessary compounds that regulate the physiology of the biofilm community. Persister cells, phenotypic variants of the wild type, will neither grow nor die in the presence of a drug. Despite their small number (1%) in the biofilm, persister cells are important in pathogenesis. During aggressive antimicrobial treatment, persister cells will survive. When the antimicrobial agent is removed, the persister cells can then give rise to normally growing cells. These cells may be responsible for some recurrent infections.

Gene transfer and expression

A biofilm is an optimal environment for the horizontal transfer of genetic material (i.e., DNA). The microorganisms live in close proximity; this facilitates the uptake of DNA by transformation and conjugation. Plasmids, for example, are expensive for cells to maintain unless they offer a selective advantage. If a plasmid is lost by one cell or DNA is released from a cell because of lysis, a nearby cell may take it up, a process termed *transformation*. The horizontal gene transfer can lead to the spread of antimicrobial resistance within the community.

Upregulation to the biofilm phenotype has been associated with the clustering of genes associated with virulence. Some studies have indicated that the biofilm is a virulence factor and should be regarded as a means whereby a disease process is enhanced. Endotoxin and the production of extracellular enzymes by the community are controversial, but there is no question that enzymatic activity for certain cohabitants is increased and that there is no universal growth of the biofilm on an inanimate surface.

Disaggregation

Disaggregation or detachment of aggregates clearly has the potential of transmitting already upregulated resistant aggregates of microorganisms to other body sites. Like cancer metastasis, this is most significant when aggregates spread via the bloodstream. Disaggregation provides the microorganisms with an opportunity to reach almost any body site. Important to disaggregation is the resulting planktonic phenotype to biofilm phenotype ratio. As the ratio increases, the risk of the microorganisms spreading increases, leading to increased risk of more serious disease. There is some evidence, particularly in ventilator-associated pneumonia, that this ratio is a direct reflection of the disease process.

Case check 31.2

Despite antimicrobial therapy, the patient in the Case in Point experienced chronic VRE infection and septicemia. The biofilm produced on the prosthetic left knee joint afforded the bacteria protection from the host's defenses and antimicrobial therapy. The sepsis likely originated from disaggregated cells dislodged from the biofilm.

Diseases associated with biofilms

Medically, the primary location of biofilms is on IMDs, but they also exist in human tissue. An estimated 80% of all human infections are attributed to biofilms. They have been linked to vaginitis, colitis, gingivitis, conjunctivitis, urethritis, highly persistent wound infections, middle ear infections, and other infections. Biofilms may be one of the leading causes for a shift from acute-phase diseases to chronic and recurrent diseases. Hence, as we transition to an older population with more chronic infections and IMDs, it is important that the laboratory scientist consider this population and associated biofilm diseases. The list of human diseases associated with biofilms has grown significantly in the past several years and involves more than 30 bacteria and yeasts. [Table 31.3](#) lists several medical or dental biofilm-associated conditions. Diseases associated with biofilms include rhinosinusitis and soft tissue disease, particularly that of dystrophic epidermolysis bullosa. Clinical studies have recognized that nontuberculous mycobacteria can form biofilms on the human cornea, resulting in vision loss. The spirochete *Borrelia burgdorferi*, the causative agent of Lyme borreliosis (disease), forms biofilms that enhance its pathogenicity. It is possible that biofilm formation contributes to the chronic nature of Lyme disease. During chronic Lyme disease, the bacteria become more tolerant to antimicrobial therapy, and scientists have demonstrated the presence of persister cells in this situation. In almost all cases of chronic infections, the biofilm plays a key role in helping microorganisms survive or spread within the host.

Dental biofilms

Dental biofilms, more commonly called *plaque*, are probably the most studied natural human biofilm. The microorganisms

Table 31.3 Partial list of human infections involving biofilms

Infection or disease	Common bacterial species
Bacterial vaginosis	<i>Gardnerella vaginalis</i> , <i>Atopobium vaginae</i> , and other species
Biliary tract infections	Enteric bacteria
Cystic fibrosis	<i>Pseudomonas aeruginosa</i>
Dental caries	Various species
Endometritis, chronic	<i>Enterococcus faecalis</i> , <i>E. coli</i> , <i>G. vaginalis</i> , and other species
Infectious kidney stones	Gram-negative bacilli
Lyme disease	<i>Borrelia burgdorferi</i>
Musculoskeletal infections	Gram-positive cocci
Native valve endocarditis	Viridans group streptococci, staphylococci, and enterococci
Necrotizing fasciitis	Group A streptococci
Osteomyelitis	Various species
Otitis media	Nontypeable <i>Haemophilus influenzae</i> , <i>Streptococcus pneumoniae</i> , <i>Moraxella catarrhalis</i>
Periodontal disease	Various species
Rhinosinusitis, chronic	<i>Staphylococcus aureus</i>
Typhoid fever, chronic	<i>Salmonella</i> Typhi
Wounds	Various species
Infections associated with foreign-body material	
Contact lenses	<i>Pseudomonas aeruginosa</i> , gram-positive cocci
Sutures	Staphylococci
Artificial heart valves	Staphylococci
Vascular grafts	Gram-positive cocci
Arteriovenous shunts	Gram-positive cocci
Endovascular catheter infections	Staphylococci
Peritoneal dialysis peritonitis	Various species
Urinary catheter infections	<i>Escherichia coli</i> , gram-negative bacilli
Intrauterine devices	<i>Actinomyces israelii</i> and other species
Penile prostheses	Staphylococci
Orthopedic prostheses	Staphylococci

*Biofilm formation in the gallbladder.

making up a dental biofilm are considered normal biota of the oral cavity. Plaque can lead to caries (dental cavities); gingivitis, a mild and reversible form of periodontal disease; and periodontitis, inflammation of deep tissue below the gum surface that can result in tooth loss. In addition, epidemiologic studies have linked dental plaque to cardiovascular disease. The rate of cardiovascular disease is 25% to 50% higher in patients with periodontitis than in healthy individuals. Atherosclerosis of coronary arteries begins as an inflammatory process involving the endothelial cells in the blood vessel wall. Macrophages, T cells and B cells, and mast cells accumulate.

Oral bacteria can access the bloodstream during gingivitis and colonize the coronary plaque, contributing to atherosclerosis by stimulation of an innate immune response. Damage to the periodontal tissue by the oral plaque bacteria can lead to release into the bloodstream of numerous cytokines (e.g., tumor necrosis factor alpha, interleukin-1, cathelicidin, and C-reactive protein) that might contribute to the atherosclerotic process in the coronary arteries. In addition, poor oral hygiene and gum disease can allow oral microbiota to reach the bloodstream. Normal oral microbiota associated with dental plaque, such as *Aggregatibacter actinomycetemcomitans*, *Eikenella corrodens*, *Streptococcus* spp., *Capnocytophaga*, *Neisseria*, and *Lactobacillus*, have been linked to infectious endocarditis.

Development of dental biofilms follows a sequence of events and involves hundreds of bacterial species. After a dental cleaning at the dentist's office, tooth enamel becomes coated with a variety of proteins and glycoproteins of host origin. This coating develops almost immediately and is referred to as the *acquired pellicle*. After pellicle formation, the primary colonizers, first streptococci (particularly the sanguinis-group streptococci) and later actinomycetes, colonize the surface of the teeth. Typical bacterial adhesion molecules and pili play a role in attachment to the pellicle. The bacteria can remain attached, despite shearing forces of saliva and tongue movement.

The bacteria on the pellicle form cell-to-cell interactions (aggregates or coadhesions), with the same species and with different species. The sessile organisms undergo cell-to-cell communication via QS, initiating changes in gene expression. Notably, several streptococci, including *Streptococcus mutans* and related organisms, begin to synthesize insoluble glucan polymers used in the production of a glycocalyx, an EPS. Bacteria from several species can bind to glucan via glucan-binding proteins. Bridge bacteria, including members of the genus *Fusobacterium*, form aggregates with the primary colonizers. The bridge bacteria cannot bind to the pellicle but can bind to the primary colonizers. The primary colonizers cannot directly form aggregates with the late colonizers. The late colonizers forming aggregates with the fusobacteria include *Streptococcus salivarius*, propionibacteria, *Prevotella*, *Veillonella*, and *Selenomonas flueggei*. At this point, the biofilm consists primarily of nonpathogens. However, in the presence of dietary sucrose and other carbohydrates, acids are produced via fermentation. The production of organic acids on the tooth surface can result in demineralization of the tooth enamel and, over time, caries.

At the base of the tooth between the tooth and gingiva (gum) is a crevice called a *sulcus*. The plaque can extend into this space and is referred to as *subgingival plaque*. In immunocompetent individuals with good oral hygiene, the plaque remains composed primarily of gram-positive bacteria. This condition is not generally associated with disease because of a balance between host defenses and bacterial growth. If the subgingival plaque is allowed to remain undisturbed for several days, the microbiota changes and becomes composed mainly of anaerobic and facultative anaerobic, gram-negative bacilli and spirochetes.

The last colonizers of the biofilm are considered pathogenic because of their role in periodontal disease. The most important pathogens include *Porphyromonas gingivalis*, *Bacteroides forsythia*, *Aggregatibacter actinomycetemcomitans*,

and the spirochete *Treponema denticola*. These organisms require the presence of late colonizers to attach to the biofilm. The pathogens produce several virulence mechanisms, allowing them to adhere to the epithelium lining the gums and invade the tissue, triggering an inflammatory response. PMN cells arrive and degranulate; the contents of their granules enhance inflammation. Cell-mediated and humoral-mediated immune responses also contribute to destruction of host tissue.

Initially, the inflammation, called *gingivitis*, is reversible and relatively mild. If the disease is allowed to progress because of poor oral hygiene and lack of medical intervention, the inflammation can extend to the periodontal support structures (ligaments, cementum, and alveolar bone), resulting in periodontitis. If the disease continues to progress, it can result in irreversible destruction of the ligaments and alveolar bone that hold the teeth in place, causing loosening of the teeth, pain, difficulty chewing, and eventually tooth loss. Gingivitis and mild periodontitis are considered relatively common. Periodontitis follows gingivitis, but it is not inevitable that periodontitis will develop following gingivitis.

Cystic fibrosis biofilm

CF is an important disease for which there is strong evidence that the chronic lung infection is the result of a biofilm. A variety of bacteria and fungi can be involved in the biofilm. Some bacteria that have been implicated include nontypeable *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *S. aureus*; the most predominant fungi are *Aspergillus fumigatus* and other *Aspergillus* spp. Infections with these bacteria are generally more amenable to treatment than the more common infections caused by *P. aeruginosa*, *B. cepacia*, *Stenotrophomonas maltophilia*, and *Achromobacter xyloso*. Up to 80% of patients with CF are chronically infected with *P. aeruginosa* by adolescence, and *P. aeruginosa* is the cause of death in most of this patient population. *P. aeruginosa* is an opportunistic human pathogen, meaning that this bacterium more commonly infects individuals with compromised host defenses. The bacterium is found in the environment in a variety of niches, such as in soil and water and on plants. In the hospital and long-term health care facility environments, the bacterium is often found in sinks, toilets, and floor mops, and it has been found in disinfectants. It causes a variety of infections because individuals in these facilities have compromised host defenses.

During infections, *P. aeruginosa* originally exists as planktonic bacteria or in enclaves of bacteria in which the bacteria later form a biofilm. In patients with CF, *P. aeruginosa* is thought to bind to the thick, viscous mucus commonly found in this patient population, forming aggregates. The bacteria frequently become mucoid from the production of an alginate slime layer. This mucoid material contributes to the extreme viscosity of the sputum produced by these patients. The change to the mucoid phenotype may allow the bacteria to increase their defenses against antimicrobial agents and host defenses. PMN cells are recruited to the biofilm and degranulate, causing tissue damage and provoking inflammation. The persistent inflammation, accompanied by bacterial toxins, contributes to the release of DNA, which has been shown to facilitate the formation of

biofilms. The inflammatory response produces most of the damage to the lungs, and over time, the result is respiratory failure.

There have been great strides in the treatment of *P. aeruginosa* infection in patients with CF. Daily inhalation of antimicrobial agents, such as tobramycin and colistin, is currently added to oral treatment regimens of ciprofloxacin, or intravenous (IV) antimicrobials consisting of a combination of an aminoglycoside and β -lactam are used. The use of inhaled antimicrobial agents allows the delivery of greater concentrations (100 to 1000 times) to the infected site than IV administration of the same drug. However, despite this intensive treatment of *P. aeruginosa* infections, the bacterial biofilm is never fully eradicated from the lungs. The intensive treatment does improve breathing and reduces the lung damage provoked by the infecting bacteria. Before the 1970s, about 50% of patients with CF would survive for 5 years; today, individuals can survive for decades.

Studies indicate that specific components of the host likely play a key role in the formation of biofilms in the CF lung. Therefore host factors, along with features of the infecting bacteria, must be considered when assessing the efficacy of new treatments. Although *P. aeruginosa* is the predominant bacterium associated with the biofilm found in adolescent patients with CF, evidence suggests that the biofilm consists of a variety of bacteria and fungi. However, the interaction of these various microorganisms in the biofilm is far from understood. The prevention or reversion of biofilm development likely would improve the quality of life of patients with CF.

Chronic upper respiratory tract infections

Studies have indicated that biofilm formation on the tonsillar tissue and adenoids can lead to chronic infections of the oral cavity and upper respiratory tract. Recurrent infections, most commonly by *S. aureus*, *Haemophilus* spp., and *Streptococcus* spp., result in hypertrophy of the tonsillar or adenoid tissue. There seems to be a link between the presence of biofilms and chronic inflammation. These biofilms might be a source of chronic pharyngitis and laryngitis. In addition, children with chronic otitis media with effusion (OME) were found to have biofilms covering part of the adenoids with OME pathogens. Similar results were found in children with chronic rhinosinusitis. When oral epithelial cells are covered with biofilms of *Streptococcus oralis* and *Streptococcus salivarius*, the cells are protected from colonization by *Streptococcus pyogenes*, a common cause of pharyngitis.

The changing paradigm of microbial infections

The understanding of chronic infections caused by organisms growing as biofilms has become more important. The role of IMDs cannot be underestimated in infections. IMDs are abiotic surfaces to which there is a natural aggregation of organisms that have a predilection for biofilm formation and the phenotypes associated with multispecies aggregates. [Table 31.4](#) lists the microorganisms associated with biofilms on IMDs. [Table 31.5](#) lists the usage and infection risk associated with IMDs in the United States, and the list is growing. In the area of dentistry alone, the use of implants and the growing treatment of patients without teeth will have a significant

Table 31.4 Microorganisms commonly associated with biofilms on indwelling medical devices

Microorganism	Indwelling medical device
<i>Candida albicans</i>	Artificial voice prosthesis
	Central venous catheter
	Intrauterine device
Coagulase-negative staphylococci	Artificial hip prosthesis
	Artificial voice prosthesis
	Central venous catheter
	Intrauterine device
	Prosthetic heart valve
	Urinary catheter
<i>Enterococcus</i> spp.	Artificial hip prosthesis
	Central venous catheter
	Intrauterine device
	Prosthetic heart valve
	Urinary catheter
<i>Klebsiella pneumoniae</i>	Central venous catheter
	Urinary catheter
<i>Pseudomonas aeruginosa</i>	Artificial hip prosthesis
	Central venous catheter
	Urinary catheter
<i>Staphylococcus aureus</i>	Artificial hip prosthesis
	Central venous catheter
	Intrauterine device
	Prosthetic heart valve

Table 31.5 Risk-related infections of indwelling medical devices in the United States

Device	Usage	Infection risk (%)
Bladder catheter	Tens of millions	10–30
Cardiac-assist devices	700	50–100
Cardiac pacemakers	400,000	1–5
Central venous catheters	5 million	5–8
Dental implants	1 million	5–10
Fracture fixators	2 million	5–10
Joint prostheses	600,000	1–3
Penile implants	15,000	2–10
Prosthetic heart valves	85,000	1–3
Vascular grafts	450,000	2–10

impact on the number of biofilm-associated dental infections beyond caries and periodontitis.

Ventilator-associated pneumonia, a condition that consumes more resources in the intensive care unit than any single infectious disease, is another example in which biofilms are an important disease-contributing factor. Pneumonia is the second most frequent hospital-acquired infection, and

86% of hospital-acquired pneumonias are ventilator-associated. Nearly 300,000 cases occur annually in the United States. The stress and shape of the endotracheal tube affect the cellular aggregation and fitness of the luminal biofilm, whether it is in the region closest to the ventilator, in the midsection (where the greatest turbulent stress is found), or in the section closest to the lung-endotracheal interface.

There are “good” and “bad” biofilms. In the human ecosystem, there is estimated to be more than 10^{14} bacteria. A hypothesis is that ecologic pressure is necessary for low numbers of pathogens to outcompete normal microbiota and achieve numeric dominance associated with diseases. Biofilms of stable normal biota represent a baseline organization. Biofilms of pathogenic organisms that overgrow normal biota and less virulent organisms because of selective pressures from antimicrobial agents are often associated with mismanagement. Microorganisms that are outcompeted often have low affinity for developing the biofilm phenotype. It is also important to remember that biofilm composition is reflective of the normal biota and location of IMDs placed close to the four reservoirs of normal biota: the gastrointestinal tract, genitourinary tract, oral cavity, and skin.

Laboratory consequences associated with biofilms

Historically, laboratory protocols focused on the idea that free-floating microorganisms were the most clinically significant. Clinical laboratories have maximized techniques that support the growth of planktonic organisms. However, broth cultures will not always facilitate the recovery of biofilm phenotypes, given that environmental conditions are required to convert a sessile phenotype to a planktonic phenotype. A broth culture incubated at 35°C with complex nutrients may not select for upregulation of an organism community. With greater understanding of biofilms and their significance in infections, better practices in the clinical microbiology laboratory must be developed.

With the emergence of biofilm-associated diseases, considerable diagnostic problems exist for the clinical laboratory. In general, these problems can be classified into five categories: (1) false-negative cultures, (2) viable but nonculturable organisms, (3) underestimated or low colony count, (4) inappropriate specimen, and (5) loss of or decreased antimicrobial susceptibility. Biofilms are resilient, adherent, and with EPS, resistant to culturing by swabs. False-negative culture results can occur because of improper sample collection. Studies have shown that cultures detect less than 10% of molecularly detectable methicillin-resistant staphylococci from vaginal specimens of biofilm communities. Some persister cells found in biofilms are viable but nonculturable, for reasons not fully understood. Organisms upregulated to the biofilm phenotype may have significantly altered biochemical profiles. These organisms will not grow under normal laboratory conditions. They may be visible on Gram-stained smears but might not be grown in culture media.

When bacteria are recovered from a biofilm, often aggregates representing 10^5 cells/mL or more can be recognized as a single colony on a bacteriologic plate. Hence the colony count may be significantly falsely reduced. Ignoring the

presence of a biofilm can result in the collection of inappropriate specimens. Until the true nature of biofilms was understood and the importance of stress realized, most organisms could be considered attached (as in arterial line sepsis) on the outside of the inserted line. It is now apparent that bacteria preferentially select a luminal environment, often devoid of immunologic components. The only technique that has been associated with the recovery of organisms from extraluminal sources is the rolling (Maki) technique. This is a semi-quantitative culture technique in which a 4-cm length of the catheter is rolled over the surface of an agar plate. Recovery of more than 15 colonies along with the same organism isolated from peripheral blood, with clinical signs and symptoms and no other recognized source of the organism, is suggestive of a catheter-related bloodstream infection. Currently, it is recognized that line sepsis is often intraluminal and that procedures must be developed to recover luminal organisms attached as biofilms. Central line-associated bloodstream infections are an important cause of morbidity and death; the Centers for Disease Control and Prevention (CDC) estimates that they contribute billions of dollars annually to U.S. health care costs.

Microbiology laboratories historically have performed antimicrobial susceptibility testing on organisms in the planktonic phenotype state in pure culture. This is associated with expressed gene markers and detection of drug resistance of an organism. In contrast, a community of cells loses its susceptibility as a phenotypic expression of that community. It is now accepted that the minimum inhibitory concentration against a free-floating planktonic isolate will increase by 10- to 1000-fold when measured in the biofilm community. Drug resistance is amplified even more when multiple species are present, particularly with the presence of prokaryotic and eukaryotic cells. Consequently, bacterial isolates that appear susceptible *in vitro* will not respond to therapy or can relapse several months later because of the existence of biofilms *in vivo*. Biofilm colonization of IMDs often requires removal of the device for a successful outcome.

Detection of biofilms and *in vitro* formation

The direct detection of biofilms on biopsied tissue, such as the middle ear mucosa in patients with chronic otitis media and on tissue or implants removed from diseased areas of the body, is now possible. Detection is based on the use of combined methods, such as polymerase chain reaction assay and pathogen-specific probes, along with special microscopic methods such as confocal laser scanning microscopy (CLSM). Although not performed routinely in clinical microbiology laboratories, these techniques provide valuable information about the role of biofilms in the disease process and what interventions are possible to eliminate or prevent biofilms.

Recently, our knowledge about the organisms that form biofilms has increased, allowing us to learn how to prevent them from initiating biofilm formation and to eliminate them. Methods used include observations by microscopic techniques (e.g., epifluorescence microscopy, CLSM, transmission electron microscopy, scanning electron microscopy)

or enumeration of sessile bacteria after detachment from the surface by scraping, vortexing, sonication, or the use of beads. These methods provide detailed information, but they are laborious and time-consuming and thus do not lend themselves to large-scale screening assays. Therefore more rapid and less laborious methods have been devised to evaluate the potential for bacteria to form biofilms in tubes or microtiter wells.

One rapid method is a colorimetric assay in which a suspension of bacteria is prepared in a culture medium that is then placed in the wells of a microtiter plate or tubes. After incubation for 24 to 48 hours, the medium is removed from the wells, and each well is rinsed several times to remove any bacteria not adhering to the vessel. Next, a stain, such as crystal violet or safranin, is added. After a specified time, the wells are rinsed several times, and then the wells are evaluated with a spectrophotometer for the presence of stain. The ability of the bacteria to form biofilms is determined by the degree of stain adhering to the bacteria in the vessel after rinsing. Techniques like this allow study of the effects an antimicrobial agent might have on the formation or inhibition of the growth of a biofilm. In a similar method, bacteria are incubated in the presence of silicone disks, and the ability of the bacteria to adhere to the disks is determined by a colorimetric method. Enhancement of this technique using preferential stains allows the determination of live and dead bacteria within the biofilm. The main problem with assays for the detection of biofilms or the potential of bacteria to form biofilms is the lack of standardization; therefore analysis of data from these studies must include a critical assessment of the methods used.

Potential interventions

Because of the inherently resistant nature of biofilms to antimicrobial therapy, biofilm inhibition strategies have been investigated. IMDs and biomaterials are ideal for microbial attachment. A common strategy to resist biofilm formation is to alter the composition of the device or its surface by adding a coating that is antiadhesive or repels attachment. Alternatively, applying low-molecular-weight biofilm inhibitors (e.g., phenol, imidazoles, furanone) has shown promise.

Traditional antimicrobial therapy based on planktonic susceptibility profiles will have a limited effect on resilient multispecies biofilms because of the physical and chemical properties of biofilms. Therapeutic modalities are focusing on multiple interventions, recognizing that the properties of biofilms resemble those of organic polymers, not planktonic microbes.

Because dispersed cells are generally more susceptible to antimicrobial treatment than biofilm-residing cells, biofilm dispersal has been investigated. Techniques to disperse biofilm are often based on interfering with metabolic pathways such as QS. A group of compounds referred to as **quorum-sensing inhibitors** (QSIs) or *quorum-quenching compounds* have been studied. These agents target bacterial signaling molecules, signal biogenesis, or signal detection, which disrupt the biofilm. An *in vitro* *P. aeruginosa* biofilm model found that a QSI used with an antimicrobial agent (ciprofloxacin, tobramycin, or colistin) had a statistically significant effect on biofilm reduction compared with

treatment with an antimicrobial agent alone. Also, the use of monoclonal antibody cocktails directed against the family of DNABII proteins has shown some success in disrupting biofilms. This disruption occurs because DNABII proteins serve as lynchpin proteins, positioned at the vertices of the cross-strands of extracellular DNA within the biofilm. Disruption of these proteins results in destabilization of the biofilm, causing it to disaggregate. Disruption of the biofilm and its aggregates could have clinical consequences that are detrimental to the patient. Disaggregation of upregulated phenotypes could be associated with metastasis. Therefore dispersal methods should be used in conjunction with antimicrobial agents.

Biofilm eradication agents are stand-alone antimicrobial agents that target and eliminate biofilms. In addressing biofilm eradication, various strategies have been tested: (1) antimicrobial agents, (2) mechanical disruption or removal (sonication), and (3) immune modulation. Other schemes to address biofilm-associated diseases have had limited success. Antimicrobial peptides are some of the most studied types of biofilm eradication agents. They have a molecular weight of 1 kDa to 5 kDa and are typically cationic (positive charge). Their mode of action is not well understood, but they disrupt cytoplasmic membranes and inhibit protein folding or enzyme activity. LL-37 was one of the first compounds to demonstrate the ability to reduce the thickness of biofilms. Oritavancin, a semi-synthetic lipoglycoprotein, has also shown promise.

Quaternary ammonium compounds are a large class of antimicrobial agents also investigated as biofilm eradicating agents. They disrupt bacterial plasma membranes, leading to metabolite leakage and eventually cell lysis. Several other compounds, including antimicrobial lipids (fatty acids), anti-cancer drugs (mitomycin C and cisplatin), phenazines, and quinolones, have been investigated.

The effects of various antimicrobials, used alone or in combination, on the elimination or reduction in the number of organisms in a biofilm or the prevention of biofilms have been studied. For example, a comparative analysis of the activities of daptomycin, linezolid, minocycline, tigecycline, and vancomycin used alone or in combination with rifampin against catheter-related methicillin-resistant *Staphylococcus aureus* (MRSA) bacteremic isolates embedded in biofilm was performed. The study found that when used alone, minocycline, daptomycin, and tigecycline were more efficacious in inhibiting the MRSA in the biofilm than linezolid, vancomycin, and the negative control. Daptomycin by itself was found to be the fastest in eradicating the MRSA biofilm. Rifampin alone was not effective in eliminating the MRSA biofilm; however, when it was used in combination with other antimicrobials, it enhanced the performance compared with when antimicrobials were used alone. This study illustrates the effects antimicrobials might have on a monospecies biofilm. No generalizations can be made, however, about the effect of any one antimicrobial on biofilms formed by multispecies biofilm.

Another approach to eliminating biofilms uses bacteriophages genetically engineered to express a biofilm-degrading enzyme called *dispersion B* (DspB) to attack the bacterial cells in the biofilm and biofilm matrix (i.e., EPS) simultaneously. Initial studies showed that the engineered phage achieved 99.9% removal of a biofilm produced by *E. coli* on plastic

pegs. This approach eliminates the need to express, purify, and deliver large doses of enzyme to specific sites of infection, all of which can be difficult. This approach is an example of synthetic biology, which over the years has enabled the development of many engineered biological devices. Synthetic biology is distinguished from traditional genetic engineering by using modularity, abstraction, and standardization to allow generalized principles and designs to be applied to different scenarios. This is an example of applied microbiology.

Biofilm models for studying antimicrobials

Establishing biofilms in 96-well plates has allowed a quantitative measurement of biofilms termed *minimal biofilm elimination concentration*. This assay quickly screens biofilms for drug resistance. In theory, disk diffusion antimicrobial testing should be related to antimicrobial-resistance testing, and biofilm testing or colonization resistance can be associated with antimicrobial susceptibility testing.

Numerous methods have been devised for studying the effects of antimicrobials on biofilms, including animal and human in situ models. The ethical implications for conducting animal and human studies must be considered carefully. Many complexities must be evaluated when devising methods or designing an apparatus for antimicrobial testing of biofilms. For example, it has been shown that the way bacteria associate with the surface influences the susceptibility of the biofilm to antimicrobials. Some of the biofilm models that have been used include:

- CDC Biofilm Reactor. Biofilms are grown on coupons (stainless steel or porcelain disks) exposed to a continuous culture, with the coupons then exposed to antimicrobials.
- Modified Robbins cell. This is like the CDC Biofilm Reactor, except that the coupons can be removed from the device and exposed to individual antimicrobials.
- Flow cell. Biofilms are grown in a continuous or batch culture with direct visualization of the biofilm possible; the biofilms can be exposed to antimicrobials.
- Membrane filters. Biofilms are grown on the surface of a membrane filter placed on an agar surface, which can then be exposed to antimicrobials.
- Microtiter plate. Biofilms are grown on plastic pegs; the pegs with the biofilm are placed into different concentrations of antimicrobials.

Do all members of a microbial community have to be grown in pure culture and tested individually, or is it sufficient to grow and test a mixed culture? Because modern molecular techniques have demonstrated that microbial communities affect disease outcome, the need to incorporate microbial communities into disease diagnosis and treatment has become ever so important. There is a need for the scientific community to consider infectious disease causation in a broader systems biology context. Host genetic variability, health status, and past exposure to antimicrobials and perhaps to other drugs and microbes individually and in communities now must be considered if the appropriate clinical response to treatment is to be achieved.

POINTS TO REMEMBER

- All microorganisms, including prokaryotes and eukaryotes, can exist in two phenotypes: planktonic and sessile.
- Biofilms likely evolved as a means of increasing microbial survival.
- Biofilms have unique properties, which are described and quantified by physical and chemical measurements, including rheology and viscoelasticity.
- Pathogenicity associated with biofilms is not a total of the individual phenotypes but rather a combination of the entire community of cells. Expression of disease is particularly associated with immunologic and inflammatory cascade and resultant tissue injury, with limited microbial injury.
- The staging of biofilms is critical in disease progression; the ratio of biofilm to planktonic phenotypes is a predictor of virulence or pathogenicity.
- Biofilms are innately more tolerant to antimicrobial agents than their individual planktonic counterparts.
- The presence of IMDs increases the risk for biofilm formation and subsequent infection.
- Many infections in animals follow monospecies or multispecies biofilm formation.
- Interventions against biofilms include treating IMDs to prevent biofilm formation and using biofilm eradication agents and combination antimicrobial agents.
- Biofilms are not recovered or recognized by standard laboratory protocols. Routine susceptibility testing does not accurately predict the success of antimicrobial therapy for biofilm phenotypes.

LEARNING ASSESSMENT QUESTIONS

- Which of the following surfaces is most susceptible to biofilm formation?
 - Liquid
 - Rough
 - Smooth
 - Metal
- At which location in a biofilm are most persister cells likely to be found?
 - Surface
 - Middle layer
 - Aqueous layer
 - Bottom layer
- What is the sticky matrix that makes up the majority of the nonaqueous portion of a biofilm?
 - Sialic acid
 - Cathelicidin
 - Exopolysaccharide
 - N-acyl homoserine lactone
- What is the first step in biofilm formation on the surface of teeth?
 - Glycolyx synthesis
 - Pellicle formed from host proteins
 - Release of quorum-sensing inhibitors
 - Bacterial teichoic acid binding to tooth enamel
- How does the planktonic phenotype differ from the sessile phenotype?
 - The planktonic phenotype often expresses pili, and the sessile phenotype does not.
 - The sessile phenotype is adapted for free-swimming, and the planktonic phenotype is attached.
 - The planktonic phenotype often produces exopolysaccharide, and the sessile phenotype does not.
 - The planktonic phenotype disaggregates forming a new biofilm, and the sessile phenotype does not.
- What is the primary trigger for inflammation in gingivitis?
 - Acid production by bacteria on tooth surface
 - Hypersensitivity reaction to bacterial allergens in a biofilm
 - Bacterial toxins released by bacteria in a biofilm in the sulci
 - Bacterial toxins released by bacteria in a biofilm on the tooth surface
- Recurrent otitis media with effusion has been linked to biofilm formation on which of the following?
 - Adenoids
 - Epiglottis
 - Nasopharynx
 - Trachea
- What is the primary disadvantage of using the rolling (Maki) technique for culturing arterial catheter tips?
 - Low sensitivity
 - Prone to contamination
 - Requires prolonged incubation
 - Only recovers extraluminal bacteria
- Which bacterium is a significant cause of morbidity and mortality in patients with cystic fibrosis as a result of biofilm formation?
 - Bordetella parapertussis*
 - Escherichia coli*
 - Pseudomonas aeruginosa*
 - Staphylococcus aureus*
- What is the best strategy in controlling biofilm-associated infections related to indwelling medical devices?
 - Using treated synthetic materials that prevent biofilm formation
 - Administering a single high dose of a broad-spectrum antimicrobial agent
 - Administering multiple low doses of a broad-spectrum antimicrobial agent
 - Using organic solvents to dissolve the biofilm matrix
- Describe the five cycles of biofilm formation.
- How do biofilms aid microbial attachment to solid surfaces?
- How is a typical mature multispecies biofilm structured?
- How do biofilms increase microbial tolerance to antimicrobial therapy?
- What are the three virulence mechanisms of biofilms?
- Why do indwelling medical devices increase the risk of bacterial infection?
- What are three areas of concern for clinical microbiologists when dealing with biofilm-associated microorganisms?
- What methods can be used to study biofilm formation in vitro?
- Why does the disruption of a biofilm have serious consequences for a patient?
- How do monoclonal antibodies disrupt biofilms?

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PART

3

Laboratory diagnosis of infectious diseases: an organ system approach to diagnostic microbiology

Upper and lower respiratory tract infections

Susan M. Pacheco and James L. Cook

CHAPTER OUTLINE

General concepts of infectious diseases of the respiratory tract, 792

Anatomy of the respiratory tract, 792

Barriers to infection, 792

The role of normal biota, 793

Host risk factors, 794

Immune status of the host, 794

Seasonal and community trends in infections, 795

Empiric antimicrobial therapy, 795

Virulence factors of pathogenic organisms, 795

Upper respiratory tract infections, 796

Pharyngitis, 796

Sinusitis, 799

Otitis media, 801

Epiglottitis, 802

Pertussis, 804

Lower respiratory tract infections, 805

Bronchitis and bronchiolitis, 805

Influenza, 808

Emerging viral respiratory tract infections: coronaviruses, 809

Acute pneumonia, 811

Empyema, 817

Tuberculosis and other chronic infections, 819

Respiratory tract infections in the immunocompromised host, 823

Patients with human immunodeficiency virus, 823

Patients with other immunocompromised states, 825

Bioterrorism and respiratory infections, 827

Bibliography, 829

OBJECTIVES

After reading and studying this chapter, you should be able to:

1. Explain the mechanical defenses of each anatomic site and how alterations to these defenses may result in infectious diseases.
2. Explain the importance of normal biota in the respiratory tract and how alterations in the normal biota may result in infectious diseases.
3. Discuss the basic pathogenic mechanisms of infectious disease agents of the respiratory tract and the virulence factors that contribute to disease.
4. Describe the most common organisms causing upper and lower respiratory tract infections.
5. Describe the pathogenesis, risk factors, and complications associated with respiratory tract infections.
6. Summarize the risk factors in immunocompromised hosts that predispose them to infections and include examples of respiratory tract diseases in different types of immunocompromised hosts.
7. Recommend types of specimens that should be collected for the diagnosis of respiratory tract infections.
8. Describe the principles and methods of proper specimen collection and transport of respiratory secretions.
9. Justify the direct visual examination of respiratory samples.
10. Describe newer and emerging respiratory infections.
11. Determine the agents of bioterrorism as related to respiratory tract infections.
12. Appraise the important aspects of the diagnosis of infections of the respiratory tract through case studies.
13. Justify the increasing role of non-culture-based rapid diagnostic testing including molecular methods in diagnosing respiratory tract infections.

KEY TERMS

Acute otitis media (AOM)

Aspiration pneumonia

Bronchiolitis

Bronchitis

Community-acquired pneumonia (CAP)

Empiric antimicrobial therapy

Empyema

Epiglottitis

Healthcare-associated infection

Parapneumonic effusion (PPE)

Pertussis

Pharyngitis

Pneumonia

Sinusitis

Ventilator-associated pneumonia (VAP)

Case in point

A 52-year-old female patient came to the emergency department complaining of right-sided chest pain with each breath, a cough that produced rust-colored sputum, and fever. She reported that her symptoms began abruptly the day before, with the onset of shaking chills. Examination revealed that the patient had a fever of 102° F (38.8° C) and coarse breath sounds in the right anterior chest. The chest x-ray (see Fig. 32.5 later in the chapter) showed a right lung upper lobe infiltrate; the laboratory analysis included an elevated white blood cell count.

Issues to consider

After reading the patient's case history, consider:

1. How mechanical and functional changes in the upper and lower compartments of the respiratory tract can lead to infection
2. The importance of the respiratory tract normal biota in preventing colonization by potential pathogens and how alterations in the normal biota and colonization of normally sterile areas may lead to infection
3. How microbial virulence factors relate to the establishment and progression of infection
4. Clinical manifestations of upper and lower respiratory tract infections and the pathogenesis, risk factors, and complications of specific disease states
5. Infections in previously healthy hosts and how presentations of these infections differ in immunocompromised patients

This chapter describes respiratory tract infections from the perspective of the clinical microbiologist working with the primary care provider who must make the differential diagnosis of these infections. General concepts of infectious diseases of the respiratory tract are discussed, including the role of normal microbial biota in preventing infection and the importance of the host's immune status in determining the pathogens likely to cause disease. It outlines the anatomy of

the respiratory tract with consideration given to natural barriers to infection. The chapter proceeds with a discussion of specific respiratory tract infections organized according to anatomic site. The causes, pathogenesis, clinical manifestations, and complications of specific diagnoses are discussed, and emphasis is placed on appropriate methods of laboratory diagnosis. Respiratory tract infections in immunocompromised hosts are described. The chapter ends with respiratory tract infections caused by agents considered to be the most likely to be used in acts of bioterrorism.

General concepts of infectious diseases of the respiratory tract

Anatomy of the respiratory tract

The function of the respiratory tract is not only to perform respiration (i.e., the exchange of oxygen and carbon dioxide) but also to deliver air from outside the body to the alveoli, where gas exchange occurs. Consideration of the anatomy of the respiratory tract (Fig. 32.1) must include the entire course that air must travel—from the mouth and nose through the sinuses, into the pharynx, past the epiglottis, through the larynx, into the trachea and bronchi, and eventually into the alveoli. In addition to a role in air transport, each of these areas also plays an important role in defending the respiratory tract against infection.

Barriers to infection

The respiratory tract has many natural barriers to infection that inhaled organisms must overcome before they can cause disease. Among the elements of the respiratory tract and its functions that help prevent infection are nasal hair, mucociliary cells that line mucosal surfaces, coughing, normal

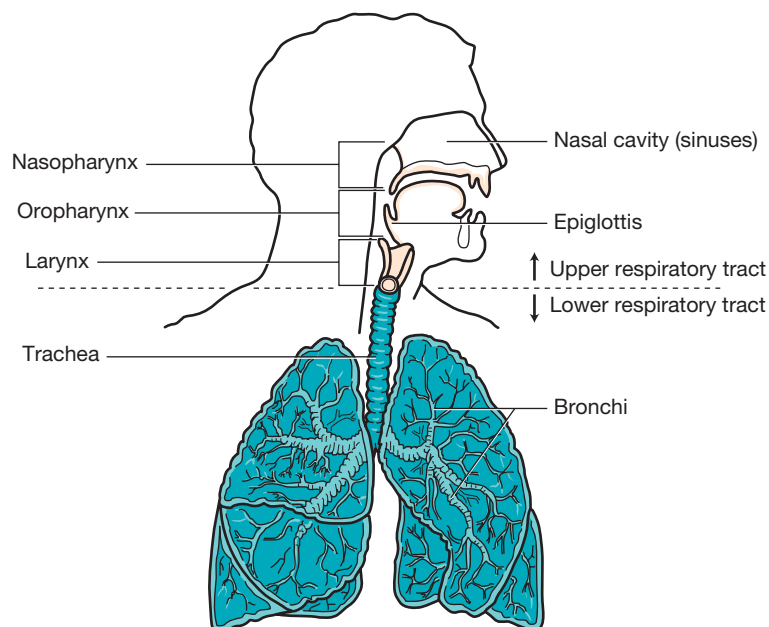


Fig. 32.1 Anatomy of the respiratory tract.

biota, secretory immunoglobulin, defensins, and phagocytic inflammatory cells.

In the nasopharynx and oropharynx, turbulent air flow causes large particles to have an impact on mucosal surfaces. Nasal hairs filter air as it passes through the nasal passages. Humidification of the air causes hygroscopic particles to increase in size, making it more likely for them to be cleared by mechanical mechanisms of the upper respiratory tract. The normal biota of the nasopharynx and oropharynx help protect the host by preventing colonization by pathogenic organisms. The mucociliary blanket of the sinuses, middle ear, and tracheobronchial tree clears particulate matter and contains immunoglobulin and other antimicrobial substances. In addition, coughing aids in the clearance and expulsion of particulate matter. If particles reach the alveoli, resident macrophages ingest microorganisms; polymorphonuclear leukocytes and monocytes are recruited once the lung becomes inflamed. Alterations in these barriers can lead to infection. For example, cigarette smoking impairs the ability of the ciliated respiratory epithelium to clear particulate matter and interferes with phagocytic cell activity. Structural abnormalities of the bronchial tree such as bronchiectasis or extrinsic compression of the bronchus by a malignancy can alter the clearance of mucus, which may contain infectious agents.

The role of normal biota

The normal microbial biota (formerly referred to as *flora*) protects the host from infection with pathogenic microorganisms. The normal biota can prevent proliferation and invasion of pathogenic organisms through competition for nutrients and the same receptor sites on host cells. In addition, some of these organisms produce bacteriocins, bacterial products that are toxic to potential pathogens. The presence of these organisms keeps the immune system primed for a rapid response to invading organisms and stimulates cross-protective immune factors termed *natural antibodies*. Under normal conditions, a balance of organisms is maintained that limits the quantity or dominance of any one type of organism.

Although a standard list of organisms can be routinely cultured from the upper respiratory tract (Box 32.1), it is interesting that the consensus concerning what is considered normal biota can change over time as new associations between organisms and disease states are recognized. For example, previously *Moraxella catarrhalis* was considered part of the normal upper respiratory tract biota that was only rarely associated with serious infections. However, since the early 1970s, it has become clear that this organism can cause respiratory tract infections in children and adults with chronic lung disease. This indicates the importance of periodic reevaluation of the pathogenicity of all organisms.

Familiarity with the normal biota of respiratory tract compartments is important in determining the clinical relevance of diagnostic testing results. For example, isolation of α -hemolytic colonies from a pharyngeal culture from a patient with pharyngitis arouses little clinical interest because α -hemolytic streptococci are normal biota in the oropharynx. In contrast, isolation of α -hemolytic colonies from a properly collected sputum specimen or bronchial aspirate in the clinical setting of lobar pneumonia should prompt full identification of the organism and perhaps initiation of empiric therapy for possible pneumococcal infection.

BOX 32.1 Normal nasopharyngeal and oropharyngeal organisms

Bacteria

Usually present

Streptococcus mitis group (including *Streptococcus pneumoniae*),
Streptococcus salivarius group, and other viridans streptococci
 Non-group A β -hemolytic streptococci
 Nonhemolytic streptococci
Veillonella spp.
Bacteroides spp.
Fusobacterium spp.
Prevotella spp.
Porphyromonas spp.
 Coagulase-negative staphylococci
Neisseria spp.
 Diphtheroids
Micrococcus spp.
Eikenella spp.
Capnocytophaga spp.

Occasionally present

Haemophilus influenzae
Haemophilus parainfluenzae
Peptostreptococcus
 Actinomycetes
Staphylococcus aureus
 Mycoplasma

Fungus

Candida spp.

Distinguishing among normal biota, colonizing, and pathogenic microorganisms

The upper respiratory tract biota in an asymptomatic patient may change, depending on the clinical setting. Patients who have previously received broad-spectrum antimicrobials, have been hospitalized recently, or have a chronic illness may have different pharyngeal biota. Gram-negative bacilli are commonly isolated from the pharynx in these patients in the absence of clinical signs of infection. Therefore it is important to distinguish between a culture positive for a potential pathogen colonizing the respiratory tract and the clinical disease state caused by that organism. Box 32.2 lists organisms that are considered primary pathogens. Also shown are a number of organisms that may or may not be pathogens, depending on the clinical setting (i.e., possible pathogens). In this case, as in many others, proper communication between the clinical microbiologist and primary care provider is essential.

Proper respiratory specimen collection increases the sensitivity and specificity of culture results. A high-quality sputum sample that minimizes contamination by oral biota and is collected before initiation of antimicrobial therapy improves the clinical value of the culture results. Interpretation of the result must be based on several factors. Characteristics of the specimen, such as the presence of white blood cells (WBCs) and number of organisms in the specimen, can help distinguish between colonization and infection. Most importantly, a compatible clinical syndrome should be present to determine whether the presence of a potential pathogen is clinically relevant. For example, isolation of a few colonies of *Staphylococcus aureus* from a sputum specimen with many

BOX 32.2 Selected nonviral pathogens in the respiratory tract**Primary pathogens**

Streptococcus pneumoniae
 Group A β -hemolytic streptococci
Staphylococcus aureus
Haemophilus influenzae
Neisseria gonorrhoeae
Bordetella pertussis
Mycobacterium avium complex
Mycobacterium chelonae-abscessus complex
Mycobacterium kansasii
Mycobacterium tuberculosis
Legionella pneumophila
 Toxin-producing *Corynebacterium diphtheriae*
Mycoplasma pneumoniae
Chlamydia trachomatis
Chlamydia pneumoniae
Pneumocystis jiroveci

Possible pathogens

Acinetobacter spp.
Pseudomonas aeruginosa
 Enterobacterales
 Fungi
Nocardia spp.
 β -Hemolytic streptococci, non-group A
Moraxella catarrhalis
 Anaerobes (e.g., *Fusobacterium necrophorum*)
 Actinomycetes

epithelial cells does not suggest *S. aureus* pneumonia but is more likely to be contamination with an organism that is part of the normal biota. In contrast, heavy growth of the same organism from a respiratory specimen with many WBCs from an older patient with postinfluenza pneumonia is highly suggestive of *S. aureus* pneumonia, especially if the sputum culture is accompanied by a positive culture from a normally sterile site, such as blood or pleural fluid.

Other organisms are potentially pathogenic and cause disease only when some interruption occurs in the pulmonary defense mechanisms. *Streptococcus pneumoniae* has been isolated from 5% to 70% of the normal adult population, yet only a very small proportion of these carriers will develop pneumococcal pneumonia. Pneumonia can also result from aspiration of upper respiratory tract secretions in patients with impaired pulmonary defenses, such as those with alcoholism or congestive heart failure. Other organisms are always considered pathogenic when isolated, even in small numbers. Isolation of *Mycobacterium tuberculosis* in any amount is significant because of the virulence of the organism and the risk for transmission from the infected patient to others.

Host risk factors**Immune status of the host**

When considering the likelihood that a microorganism will cause infection in a given host, the laboratory scientist must consider the virulence of the agent and the host defenses to counteract establishment and progression of infection. For these

purposes, a normal host is considered to be one with mature immunologic defenses who may or may not have specific immunity against the infectious agent in question. Examples of agents that cause respiratory tract infections in normal hosts include the common respiratory viruses of childhood. In normal children, these viral infections occur at a high incidence but are usually relatively benign. The clinical outcomes of these and other respiratory tract infections in normal hosts depend on the injurious effects of the microorganism and its products at the site of infection and the host's response to infection.

Previous exposure of a host to pathogens, such as the respiratory tract viruses, is one example in which the host usually develops immunity to reinfection with the same agent. Immune responses may prevent or alter the course of subsequent infection. For example, adults previously infected with a given viral serotype as children usually do not manifest the same severity of infection when reexposed to the same pathogen. Evidence of infection may not be present, or clinical signs and symptoms of infection may be greatly reduced. In an immunocompromised host, however, microorganisms that are usually not pathogens in a normal host may cause serious infection. This type of infection is referred to as an **opportunistic infection** (OI) to indicate the combination of a reduced host response and a pathogen of low virulence that results in the establishment of infection. Because the host response is diminished, infections with highly virulent pathogens are usually more severe and more rapidly progressive than in the immunocompetent host.

Age as a risk factor

Immunocompromise because of age is a form of functional immunodeficiency. Infants and older adults are more susceptible to certain infections and are more likely to develop complications of these infections. For example, respiratory tract infections with *Haemophilus influenzae* are more commonly complicated by meningitis in infants than in older children or adults. Similarly, in older adults, complications of common respiratory tract infections occur more frequently than in the younger adult population. One hallmark of seasonal outbreaks of influenza virus infection of the respiratory tract is the increased incidence of death from complicating bacterial pneumonias in older adults.

Thus it is apparent that one cannot determine the significance of a respiratory tract microbial isolate without considering the source of the specimen, age and immunologic status of the host, and clinical setting of the patient. The isolation of a potential pathogen may represent simple colonization of the upper respiratory tract or life-threatening disease. In contrast, isolation of a nonpathogenic organism that may be part of the normal biota of the upper respiratory tract may be an indication of disease if found in an unusual location or in a host with decreased defenses against infection. Availability of complete clinical data facilitates proper evaluation of the respiratory tract specimen by the clinical microbiologist.

Reduced clearance of secretions

Reduced clearance of secretions or obstruction of an area in the upper or the lower respiratory tract predisposes one to infection and can, on occasion, seriously compromise respiratory tract function. Mechanical clearance is important in limiting the numbers of potential pathogens and in the associated

inflammatory response. Decreased clearance of respiratory secretions may result from the following:

- Immature anatomic development (e.g., eustachian tube anatomy in young children)
- Transient reduction in function of the mucociliary mechanism (e.g., after viral infection or as a result of cigarette smoking)
- Obstruction by a foreign body (e.g., aspirated food or foreign object)
- Previous disease that alters the normal respiratory tract anatomy (e.g., bronchiectasis, obstructing lymph node or tumor)
- Increased viscosity of mucus (e.g., cystic fibrosis)

Infection-induced airway obstruction

Another way in which respiratory tract obstruction can be a factor in infectious disease involves compromise of respiration. In these cases, the obstruction may be a consequence of infection, rather than the factor that precipitates infection. The anatomy of the area, rather than the type of pathogen, dictates the urgency with which treatment is initiated. An example of this type of respiratory tract infection is acute epiglottitis (see later) caused by infection of a nonimmune patient with *H. influenzae*. This inflammatory response to bacterial infection can cause life-threatening upper airway obstruction and is considered a medical emergency. In contrast, other infections caused by the same pathogen, such as sinusitis, can be treated more deliberately.

Seasonal and community trends in infections

Awareness of the patterns of respiratory tract infections at different times of the year in the community in which the patient resides is important for the efficient use of diagnostic microbiology resources. Some respiratory tract infections have a peak seasonal incidence and may produce epidemics in the community. Viral respiratory tract infections, for example, are more common in the winter months. Therefore if influenza virus infection is epidemic in the community and a patient presents with symptoms and signs compatible with this viral illness, the likelihood is high that influenza is the cause of the infection. In such cases, performing extensive bacterial cultures to define the cause of the respiratory tract infection may be a wasteful use of resources unless a secondary infection is suspected.

In contrast, diseases associated with *Mycoplasma pneumoniae* typically occur throughout the year, without marked seasonal variability. The incidence of viral infections and secondary bacterial pneumonias is reduced during the summer months; therefore *M. pneumoniae* may cause up to 50% of all pneumonias in the summer months. Communication among clinical microbiologists, primary care providers, and state health department personnel sharing information on community trends in the pathogens causing respiratory tract infections helps focus diagnostic and therapeutic efforts.

Empiric antimicrobial therapy

To properly position the diagnostic microbiology laboratory in the scheme of the patient health care, it is important to understand the role of **empiric antimicrobial therapy** in the

care of patients with respiratory tract infections. Although basing antimicrobial therapy on the results of diagnostic microbiological studies is desirable, certain circumstances dictate that therapy be initiated before obtaining these results or even without submitting specimens for testing. For example, antimicrobial therapy should be initiated before obtaining microbial identification and susceptibility testing results in patients who are seriously ill with pneumonia. In some types of respiratory tract infections, it is standard to initiate antimicrobial therapy without obtaining specimens for culture, such as in children with middle ear infections (otitis media) when the pathogen can reasonably be predicted in most cases without resorting to an invasive procedure. This same rationale applies to other respiratory tract sites that are difficult to culture directly, such as the sinuses.

When empiric antimicrobial therapy is necessary, it is important to have a working knowledge of the organisms most likely to cause the type of infection observed and of the antimicrobial agents most likely to be effective. The microbiology laboratory can provide preliminary data on the possible identity of the pathogen based on Gram staining results and preliminary biochemical studies (e.g., lactose fermentation by gram-negative bacilli). Multiplex molecular diagnostic panels are also being increasingly utilized to detect common pathogens in clinical specimens including from respiratory and blood specimens within hours before the availability of culture result.

If the infection is a **healthcare-associated infection**, it is important to know whether the antimicrobial susceptibility patterns of the infectious agent in question differ from those found in the community. Periodic reviews of bacterial antimicrobial susceptibility patterns are published by clinical microbiology services to facilitate this type of decision-making process; these are termed *cumulative antibiograms*. Empiric use of antimicrobial agents and adjustment of therapy based on the results of subsequent microbiological data represent other important interactions between the primary care provider and clinical microbiologist.

Virulence factors of pathogenic organisms

The disease-producing capability of an organism is the clinical manifestation of its virulence. Microorganisms cause infection by entering the host, interacting with specific target tissues, evading the host's defenses, proliferating, damaging the host, and disseminating or elaborating products that cause systemic disease. Virulence factors involved in disease-producing mechanisms, such as adherence, toxin elaboration, and host evasion, enable the microorganism to complete this process. See [Chapter 2](#) for a discussion of host-parasite interactions.

Attachment of the bacterial pathogen to the mucus membranes is a primary step in the initiation of a respiratory tract infection. Attachment is made possible by adhesins or other microbial surface molecules or pili that bind the organism to a host surface. Specific bacterial adhesins interact with specific cellular receptors. For example, *Streptococcus pyogenes*, possess fimbriae, which are fine, irregular structures composed of M protein and lipoteichoic acid that bind to epithelial cells.

Microorganisms may elaborate toxins that produce different pathogenic effects, depending on the activity of the toxin and the target cell with which it interacts in the host. For example, *Corynebacterium diphtheriae*, the cause of diphtheria, produces a toxin that interferes with protein synthesis. Locally, the toxin induces necrosis, resulting in the formation of a pseudomembrane in the pharynx composed of necrotic respiratory epithelial cells, leukocytes, and organisms. This pseudomembrane can cause airway obstruction. Systemically, the toxin preferentially adheres to myocardial, nerve, and kidney tissue, causing myocarditis, neuritis, and renal tubular necrosis. The mechanism of action of *Pseudomonas aeruginosa* exotoxin A resembles that of diphtheria toxin, but it has a different pathogenic effect because of different target tissue and involvement of other virulence factors.

Evasion of host defenses enables microorganisms to proliferate and cause damage to the host. Certain respiratory pathogens, such as *S. pneumoniae*, *H. influenzae*, and mucoid *P. aeruginosa* strains, evade host defenses by expressing polysaccharide capsules that prevent phagocytosis by WBCs. Chlamydiae are obligate intracellular parasites taken up by host cells, where they are protected from the host immune system. *M. tuberculosis*, another intracellular pathogen, survives by inhibiting phagosome-lysosome fusion. Other respiratory pathogens are able to cleave host secretory antibodies by producing immunoglobulin A (IgA)-specific proteases.

Case check 32.1

Symptoms of acute pneumonia, as seen in the Case in Point, can arise suddenly with the patient appearing quite ill. These patients typically have a productive cough that can be blood-tinged as a result of severe inflammation occurring in the lungs. *Streptococcus pneumoniae*, an encapsulated bacterium, is the number one cause of bacterial pneumonia. The mortality rate can be high, especially in older adult patients.

Upper respiratory tract infections

Pharyngitis

Case study

An 8-year-old female patient was brought to the emergency department by her mother because the child was complaining of a sore throat and had a low-grade fever. The mother stated that her daughter had had a runny nose and cough for the past few days. On examination, the patient had a fever of 99° F (37.2° C). Her oropharynx was red, and her tonsils were slightly swollen, but no exudate was present. A neck examination revealed no tender lymph nodes.

Epidemiology

Sore throat, or **pharyngitis**, is one of the most common reasons patients visit their primary health care providers, resulting in 11 million office visits annually in the United States. Most pharyngeal infections occur in the winter and early spring. β -hemolytic

group A streptococci (GAS) and other respiratory pathogens are usually acquired by inhalation of aerosols or by contamination of the hands with droplets and then inoculation into the upper respiratory tract. In some cases, this acquisition leads to symptomatic disease, whereas in others, patients simply become asymptomatic carriers. During peak season, up to 15% of school-age children may be asymptotically colonized with GAS, which can complicate diagnosis.

Causes

Table 32.1 summarizes the clinical syndromes encountered in the upper respiratory tract and the common associated causative agents. Most pharyngitis cases in all age groups are caused by viruses, including rhinovirus, coxsackie virus, coronavirus (including severe acute respiratory syndrome coronavirus-2 [SARS-CoV-2]), adenovirus, parainfluenza virus, Epstein-Barr virus (EBV), and influenza virus. A less common but important viral cause to consider is HIV, which can manifest as a sore throat during the acute phase of the infection. The most common bacterial cause of pharyngitis is *Streptococcus pyogenes*, a β -hemolytic GAS. Identified in 15% to 30% of children 5 to 15 years of age who present with pharyngitis, GAS are isolated much less frequently (<15%) in adults and very young children with a similar clinical presentation. Other β -hemolytic streptococci, including groups C and G, are often isolated in culture. Group C streptococci have been associated with pharyngitis in college students and young adults; the role of group G streptococci is less clear.

Less common causes of pharyngitis include bacterial pathogens such as *Neisseria gonorrhoeae*, *Arcanobacterium haemolyticum*, *Corynebacterium diphtheriae*, and *Fusobacterium necrophorum*. *C. diphtheriae* causes diphtheria, a highly contagious and potentially fatal form of bacterial pharyngitis that is transmitted through close contact or inhalation of bioaerosols from infected patients. However, it is now uncommon in the United States and most of the developed world because of the widespread use of diphtheria vaccination. This infection should be suspected in patient populations in whom vaccination rates are low, such as in developing countries and potentially in underserved U.S. populations.

Pathogenesis

The reasons for the symptoms in patients with viral or streptococcal pharyngitis are incompletely understood. Some viruses (e.g., adenoviruses) that infect the pharyngeal mucosa cause cellular destruction (cytopathology); all such infectious agents elicit inflammatory cell responses. The combination of these events is responsible for the pharyngeal pain and swelling experienced by patients with pharyngitis. However, in other cases, the viral pathogens (e.g., rhinoviruses) cause symptoms of pharyngitis with little mucosal cell destruction. In some cases, these noncytopathic viruses have been shown to elicit the production of inflammatory mediators that can reproduce the symptoms of pharyngitis.

The pathogenesis of streptococcal pharyngitis may be caused mainly by the inflammatory effects of a variety of extracellular products elaborated by the bacteria. These products exhibit various activities, including toxicity to a variety of cells, pyrogenicity, and enhancement of the spread of streptococci through infected tissues.

Table 32.1 Upper respiratory tract infections

Clinical syndrome	Causative agents	Specimen collection	Other
Pharyngitis	Children: Viruses <i>Streptococcus pyogenes</i> (15%–30%) Adults: Viruses <i>Streptococcus pyogenes</i> (5%–15%)	For <i>S. pyogenes</i> culture, swab tonsils and posterior pharynx, and place in transport media; rapid strep testing available and often performed at point of care	For epidemiologic purposes, a test for SARS-CoV-2 is now commonly performed If clinically indicated: • Culture for <i>Corynebacterium diphtheriae</i> • Nucleic acid amplification test or culture for <i>Neisseria gonorrhoeae</i> • HIV RNA PCR • Monospot test for EBV
Sinusitis	<u>Most common</u> Rhinovirus Parainfluenza virus Influenza virus <u>Less common</u> <i>Streptococcus pneumoniae</i> <i>Haemophilus influenzae</i>	Direct sinus sampling as needed	Direct sampling indicated for patients who fail empiric therapy, are severely ill, are immunocompromised, or in whom intracranial extension is suspected
Otitis media	<u>Most common</u> <i>S. pneumoniae</i> <i>H. influenzae</i> <u>Less common</u> <i>S. pyogenes</i> <i>Moraxella catarrhalis</i> <i>Staphylococcus aureus</i>	Empiric therapy is used in the majority of cases Direct culture by tympanocentesis as needed	Direct culture indicated for patients who are severely ill, have failed empiric antimicrobial therapy, or have suspicion for an unusual or drug-resistant pathogen
Epiglottitis	<u>Most common</u> Streptococci Staphylococci <i>H. influenzae</i> <u>Less common</u> <i>Haemophilus parainfluenzae</i>	Blood cultures	Direct swab should be performed only if airway is secure
Pertussis	<u>Most common</u> <i>Bordetella pertussis</i> <i>Bordetella parapertussis</i> <u>Less common</u> <i>Bordetella bronchiseptica</i> <i>Bordetella holmesii</i>	Nasopharyngeal swab: • PCR testing • Plate directly onto Bordet-Gengou or Regan-Lowe medium for culture	If very late in the course of the disease, serologic testing may be more beneficial if other testing is negative

EBV, Epstein-Barr virus; HIV, human immunodeficiency virus; PCR, polymerase chain reaction.

Clinical manifestations

Clinically, differentiation between viral and streptococcal pharyngitis is difficult. Symptoms of a common cold, including rhinorrhea (watery mucus discharge from the nose), conjunctivitis, and cough, are more often associated with a viral cause; influenza virus infection is often accompanied by fever, diffuse myalgias (muscle aches), and profound fatigue.

Case check 32.2

The modified Centor criteria, which include fever, anterior cervical lymphadenopathy, tonsillar or pharyngeal exudates, and the absence of cough, are often used to risk-stratify patients for the likelihood of GAS throat infection; the more criteria present, the greater the likelihood of GAS infection. The age of the patient is also considered. As in the introductory case of the 8-year-old patient with a sore throat and low-grade fever, without swollen lymph nodes and other symptoms (rhinorrhea) more consistent with a viral infection, the use of criteria such as these is recommended to determine which patients require testing and/or empiric therapy.

Infectious mononucleosis caused by EBV or cytomegalovirus (CMV) may be suspected based on posterior cervical or generalized lymphadenopathy (swelling of the lymph nodes) and splenomegaly. Acute HIV seroconversion is also often accompanied by generalized lymphadenopathy as well as fever and a diffuse rash.

Streptococcal pharyngitis classically entails acute onset of a sore throat with tonsillar exudate, fever, tender anterior cervical lymphadenopathy, and the absence of a cough and other likely signs of viral infection. A scarlatiniform rash, if present, is more specific for GAS, although a similar rash can be seen with *Arcanobacterium* infections. Streptococcal pharyngitis may also present in a manner indistinguishable from that of viral pharyngitis. Diphtheria is classically associated with the presence of a tightly adherent pharyngeal membrane (pseudomembrane) that attaches to respiratory surfaces. *Fusobacterium necrophorum* infection has been implicated in pharyngitis, especially in adolescents and young adults; *Fusobacterium* has also been associated with inflammation and thrombosis of the internal jugular vein, with eventual embolization of the infected thrombus, a symptom complex called *Lemierre syndrome*.

Complications

The occasional complications of viral infections associated with pharyngitis usually are caused by systemic manifestations of the infections or secondary bacterial infections such as sinusitis, otitis media, or pneumonia. GAS pharyngitis can produce suppurative and nonsuppurative complications. Displacement of either or both of the tonsils or asymmetric swelling of the soft tissues of the pharynx following pharyngitis should raise suspicion of a peritonsillar or pharyngeal abscess. Although GAS have often been associated with these soft tissue infections, oral anaerobic bacteria should also be considered in the differential diagnosis. GAS pharyngitis can also result in otitis media, sinusitis, and suppurative cervical adenitis. Other suppurative complications such as meningitis are rare.

Nonsuppurative complications of GAS pharyngitis include poststreptococcal acute glomerulonephritis (immunologic injury to the kidneys), acute rheumatic fever (immunologic damage to the heart valves), scarlet fever, streptococcal toxic shock syndrome, reactive arthritis, and pediatric autoimmune neuropsychiatric disorder associated with group A streptococci (PANDAS). Rheumatic fever is now rare in most developed countries outside of outbreaks, but it remains a significant issue in the developing world. Once affected, these patients require antimicrobial prophylaxis to prevent recurrent episodes of rheumatic fever and further damage to the heart tissue. Timely diagnosis and treatment of GAS pharyngitis is the best way to prevent the development of rheumatic fever.

Laboratory diagnosis

The primary goal of laboratory testing in most cases of acute pharyngitis is to differentiate streptococcal pharyngitis from viral pharyngitis. Testing for GAS is not recommended for children and adults with pharyngitis that is likely due to a viral cause based on clinical and epidemiologic clues. In addition, testing is not recommended for most children younger than 3 years of age in the absence of an epidemiologic contact given the rarity of infection in that population. For all other patients with clinical signs and symptoms suggestive of GAS infection, testing should be performed to avoid unnecessary antimicrobial therapy in those who are not infected with GAS given the overlap in clinical presentations. Clinical prediction scores such as the modified Centor score and the FeverPAIN score are often used to guide the decision to test. It is important to note that a positive test result does not definitively diagnose streptococcal pharyngitis because some patients are asymptomatically carriers of GAS but may have a concurrent viral illness causing the symptoms. Currently, there is no accurate method to distinguish these patients from those with true infection.

Throat culture for GAS is the diagnostic gold standard and has a sensitivity of 90% to 95%. In collecting pharyngeal specimens for streptococcal cultures (Fig. 32.2), it is important to adequately swab both of the tonsillar areas as well as the posterior pharynx. If any tonsillar exudate is seen, specific efforts should be made to directly swab involved areas. The tongue and other oral structures should be avoided with the swab to minimize contamination with oral biota and dilution of the specimen. After collecting the specimen, swabs should be placed in an appropriate transport medium and cultured on nonselective media, such as sheep blood agar, incubated

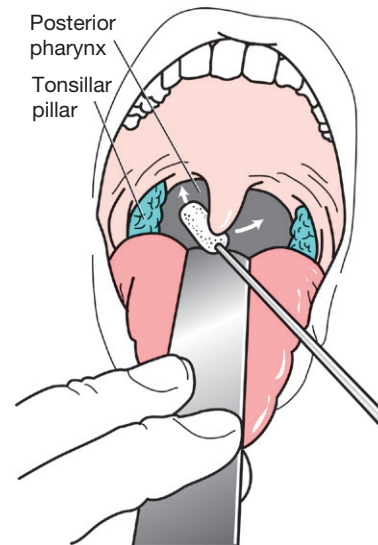


Fig. 32.2 Specimen collection from the oropharynx.

in ambient air or under anaerobic conditions. A selective medium can be added. The disadvantage of conventional culture, in contrast with rapid antigen testing, is the delay in obtaining results and the associated delay in therapy or a tendency to prescribe unnecessary empiric therapy, which might otherwise have been avoided.

Rapid antigen detection tests (RADTs) are point-of-care tests that most commonly use lateral flow enzyme immunoassays (EIAs) or optical immunoassays (OIAs) to identify GAS antigens. RADTs have excellent specificity; therefore therapeutic decisions can be made with confidence on the basis of a positive test result. The sensitivity of RADTs, however, is variable, ranging from 39% to 100% in individual studies. In children in whom the prevalence of GAS infection is higher and the risk of complications increased, it is recommended that a negative RADT result be followed up with a throat culture to avoid missing the diagnosis of GAS pharyngitis, unless the specific RADT used is sufficiently sensitive. In adults, a follow-up culture is generally not needed, given the lower incidence of GAS pharyngitis and the very low risk of developing complications, including acute rheumatic heart disease.

Serologic testing is not useful for the diagnosis of acute GAS infection but is helpful in confirming a recent streptococcal infection in cases of suspected poststreptococcal acute glomerulonephritis and acute rheumatic fever or during epidemiologic investigations. Atypical lymphocytosis and the presence of heterophile or specific IgG and IgM antibodies suggest EBV infection, and many other viruses may be detected with molecular assays (e.g., influenza virus, SARS-CoV-2). In cases of suspected acute HIV or bacterial infection, such as *N. gonorrhoeae* or *C. diphtheriae* infection, viral and bacterial polymerase chain reaction (PCR) assays should be performed.

Treatment

Treatment of viral pharyngitis is supportive, with the exceptions of influenza, HIV, and SARS-CoV-2, for which specific antiviral agents are available. Most cases of GAS pharyngitis are self-limiting, with resolution of symptoms in 3 to 4 days, even without antimicrobial therapy. Antimicrobials have been

shown to shorten the duration and, in some cases, reduce the severity of symptoms, although the effect is usually modest. Additional benefits of antimicrobial therapy include interruption of transmission of the infection to contacts and prevention of suppurative and nonsuppurative complications of GAS pharyngitis, including acute rheumatic fever. Antimicrobial therapy has not been demonstrated to prevent acute poststreptococcal glomerulonephritis. Antimicrobials should be prescribed only for episodes of proven GAS infection. Compared with reliance on clinical presentation alone, the availability of rapid testing has resulted in decreased inappropriate antimicrobial therapy for patients who likely have a viral illness; however, antimicrobials are still overprescribed for this indication.

Penicillin or amoxicillin is the drug of choice for GAS pharyngitis. Alternatives in penicillin-allergic patients include macrolides, clindamycin, and first-generation cephalosporins, depending on the specific type of allergy. Benefits of therapy for other non-GAS pharyngitis infections are uncertain. Performing a repeated throat culture or RADT after antimicrobial therapy for a test of cure is not necessary in most patients who have resolution of symptoms. The majority of patients who are asymptotically colonized with GAS do not require therapy because they generally have a low risk for developing symptomatic pharyngitis or immune-mediated complications or for transmitting infection to others. Some exceptions include a patient or close contact of a patient with a history of rheumatic fever, during rheumatic fever or poststreptococcal glomerulonephritis outbreaks, and if there are recurrent infections within a family. Patients who develop rheumatic fever warrant secondary prophylaxis to prevent subsequent GAS infections. The duration of prophylaxis depends on the clinical circumstance.

Sinusitis

Case study

A 40-year-old female patient presented to her health care provider complaining of fever and nasal discharge. She had developed cold symptoms 1 week earlier and was treating herself with over-the-counter medications, with little improvement. In fact, her symptoms had progressively worsened over the past 48 hours, with increasing headache and left facial pain. Her physical examination was notable for a low-grade fever, tenderness over the left maxillary sinus, and purulent drainage from the left side of the nose.

Epidemiology

Infectious sinusitis (or rhinosinusitis) is a common inflammatory reaction of the nose and paranasal sinuses, with approximately 30 million adults receiving a diagnosis annually. This chapter focuses on acute **sinusitis**, which is usually infectious in origin, rather than chronic sinusitis, which can have diverse causes. Cases of acute sinusitis are usually preceded by acute viral upper respiratory tract infections that occur predominantly in the winter and spring months. This disease entity is less common in children than in adults because of the incomplete development of most of the paranasal sinuses until adolescence.

Causes

Viruses, including rhinovirus, coronavirus, parainfluenza virus, adenovirus, and influenza virus, are the most common cause of acute sinusitis. Less than 2% of episodes of viral sinusitis in adults and 5% to 10% in children are thought to be complicated by bacterial infection. *S. pneumoniae* and *H. influenzae* are the bacterial pathogens historically identified as the most common causes of community-acquired infections in adults and children. *S. pyogenes*, *S. aureus*, and *M. catarrhalis* account for most of the remaining infections, the last being more common in children. In healthcare-associated infections, *S. aureus* and gram-negative bacilli are recovered more frequently.

Acute fungal sinusitis is uncommon in patients with an intact immune system; it occurs predominantly in immunosuppressed hosts treated with cytotoxic or immunosuppressive medications or in patients with uncontrolled diabetes mellitus (ketoacidosis). Respiratory allergies also predispose individuals to acute sinusitis. When recurrent infectious sinusitis is associated with recurrent pulmonary infection over prolonged periods, it suggests the possibility of cystic fibrosis, hypogammaglobulinemia, or ciliary dysfunction.

Pathogenesis

The sinuses normally undergo a continuous cleansing process through the action of ciliated epithelial cells. These ciliated cells move the mucus layer lining these areas toward the sinus ostia, the openings of the sinuses into the nasopharynx. This process normally clears the sinuses of bacteria from the adjacent nasopharynx. During acute rhinosinusitis, mucosal swelling can cause partial or complete obstruction of the sinus ostia, interrupting the flow of secretions and predisposing to bacterial overgrowth in the obstructed sinuses; this can occur with infectious and allergic causes. Viral infection and bacterial toxins can also alter the normal cleansing function of ciliated epithelial cells, and the mucus layer can become more viscous because of the resulting inflammation, further impairing this process. Noninfectious obstructions can cause a similar problem, such as foreign bodies (including nasotracheal tubes), tumors, and congenital structural abnormalities of the nasopharynx.

Clinical manifestations

In young children, the only symptoms may be persistent rhinorrhea and cough, either directly after a viral upper respiratory tract infection or following a brief period of clinical improvement. In young children with signs and symptoms of persistent sinusitis, foreign bodies in the nose must always be considered.

In adults, sinusitis is clinically suspected when purulent nasal discharge is accompanied by nasal obstruction and/or facial pain or pressure; nonspecific symptoms, including cough, headache, and low-grade fever may also be present. It is often difficult to clinically differentiate patients with viral sinusitis from those with bacterial superinfection, an issue that contributes significantly to the inappropriate prescription of antimicrobial agents for this largely viral condition. Suggested criteria for the diagnosis of bacterial sinusitis include (1) persistent symptoms without reduction for more than 10 days; (2) severe symptoms (high-grade fever, purulent

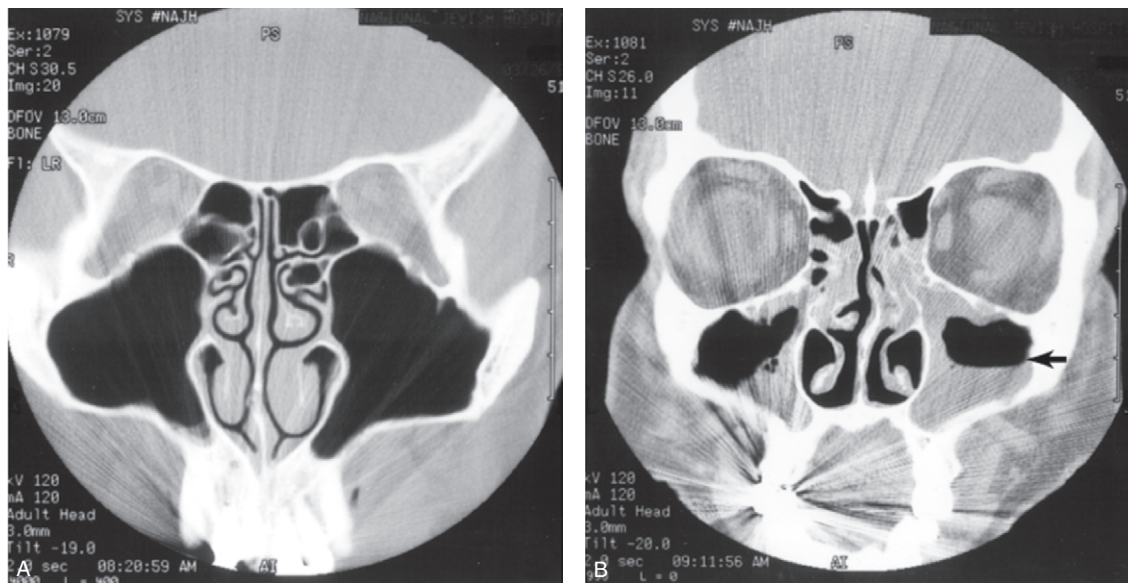


Fig. 32.3 Computed tomography scan of the paranasal sinuses in a patient with normal maxillary sinuses (**A**) and a patient with bilateral maxillary sinusitis (**B**). **A**, The large (black) cavities on either side of the nasopharynx are the normal maxillary sinuses. Of note is the absence of thickening of the lining and the absence of any opacity within the cavity of the maxillary sinuses. **B**, The difference in the maxillary sinuses compared with the image in **A** is the presence of opacification in both maxillary sinuses. This opacification represents purulence and mucosal thickening within the sinuses. The maxillary sinus on the right side of the figure also demonstrates an air-fluid level (the relatively straight, horizontal line between the air-filled black space above and the gray, fluid-filled space below [arrow]) characteristic of acute, purulent sinusitis.

discharge, and facial pain) present for 3 to 4 days at the beginning of the illness; and (3) initial reduction followed by subsequent worsening of symptoms.

Imaging with plain radiography or computed tomography (CT) scanning is not routinely recommended for the diagnosis of uncomplicated acute sinusitis because abnormalities seen on these images are nonspecific. Many patients with viral upper respiratory tract infections will have abnormal findings. Even in true sinusitis, imaging results will not distinguish between viral and bacterial causes. CT scans may be helpful, however, in patients with persistent or recurrent symptoms to identify predisposing structural abnormalities, disease complications caused by the spread of infection, or an infectious source in immunocompromised patients who have no localizing symptoms (Fig. 32.3).

Case check 32.3

The presentation of acute sinusitis in older children and adults, as in the introductory Case in Point, is often that of a prolonged respiratory tract infection that evolves into a new set of symptoms, including fever, purulent nasal discharge, and facial pain. When the maxillary sinuses are involved, facial pain may be worse when leaning forward or when a jarring force is experienced, such as walking down stairs. Patients with maxillary sinusitis can also experience headache and pain perceived to come from the upper teeth. Fever is present in only about 50% of patients with acute sinusitis.

Complications

Extension of bacterial infection to adjacent areas or structures can cause complications, including orbital cellulitis, osteomyelitis (infection of the bone), meningitis, brain abscess, and cavernous sinus thrombosis. Orbital cellulitis or retroorbital abscesses can result from direct extension

of infection from adjacent sinuses to the area around and behind the eye. Protrusion of the eye (proptosis) or limitation of ocular movements should suggest the possibility of extension of the infection to the retroorbital space; this is a serious complication that requires rapid diagnosis and emergency management.

Extension of frontal sinusitis can cause cellulitis in the forehead area overlying these sinuses and osteomyelitis of the frontal bone, with associated subgaleal abscess, referred to as *Pott's puffy tumor*. Further extension of frontal sinusitis can cause an abscess of the frontal lobes of the brain. Extension of infection from other sinuses (e.g., ethmoid, sphenoid) can involve the central nervous system (CNS) in the form of meningitis or brain abscess. Suspicion of such severe complications must remain high in patients who appear to have refractory sinusitis in the setting of altered mental status or new neurologic complaints.

Laboratory diagnosis

The gold standard for the diagnosis of sinus infection is direct sinus puncture and aspiration. This invasive and potentially painful procedure is usually unnecessary, given the knowledge available of the common etiologic pathogens. Sinus aspirate culture should, however, be considered if there is a suspicion of intracranial extension of the infection, if empiric antimicrobial therapy has failed, or if the patient is severely ill or immunocompromised. These samples are inoculated onto culture media such as sheep blood, chocolate, and MacConkey agars, which are routinely used to isolate respiratory tract pathogens. Cultures of nasal secretions or of nasal swabs are unreliable indicators of the pathogens causing acute infection within the sinus and should not be routinely performed or used for clinical decision making. For patients in whom acute fungal sinusitis is suspected, an ear, nose, and throat (ENT) specialist should be consulted immediately for

examination, obtaining a specimen for culture and histopathology, and possible debridement.

Treatment

Symptoms of viral sinusitis resolve spontaneously in 1 to 2 weeks and do not require antibacterial therapy. Despite estimations of bacterial infections occurring in less than 10% of acute rhinosinusitis cases, more than 80% of patients with a diagnosis of acute sinusitis are prescribed antimicrobial therapy, most of which is inappropriate. Antimicrobial therapy should be considered if bacterial infection is suspected based on the criteria discussed earlier (see the "Clinical Manifestations" section). For those patients with persistent symptoms that have not worsened and are not severe, some guidelines give an option to observe and reevaluate in 3 days versus prescribing antimicrobials at the initial visit.

The agent of choice in the absence of risk factors for antimicrobial resistance (e.g., age <2 or >65 years, daycare attendance, recent antimicrobial therapy, recent hospitalization, comorbidities, immunocompromised state), severe infection, or high endemic rates of penicillin-nonsusceptible *S. pneumoniae* is amoxicillin for both adults and children. If any of the previously mentioned conditions are present, amoxicillin-clavulanate is recommended. Alternatives in penicillin-allergic patients or in patients in whom treatment has failed include fluoroquinolones or doxycycline in adults and a third-generation cephalosporin plus clindamycin in children. Macrolides should be avoided because of high rates of resistance in *S. pneumoniae*. As an adjunctive therapy, intranasal corticosteroids can be beneficial for symptomatic relief in patients with a history of allergic or chronic rhinitis. Patients with healthcare-associated sinusitis may require broader-spectrum initial therapy. Patients with acute invasive fungal sinusitis require aggressive debridement and directed antifungal therapy.

Otitis media

Epidemiology

Acute otitis media (AOM), an infection of the middle ear, is the most common bacterial infection in children and one of the most prevalent reasons for visits to the pediatrician. Peak incidence is seen in children between 3 and 24 months of age, and most children have had at least one episode of AOM by 3 years of age. Identified risk factors include a history of AOM, attendance at a daycare center, family predisposition for AOM, exposure to passive tobacco smoke, nasopharyngeal colonization with otopathogens, and presence of anatomic nasopharyngeal disorders.

After the introduction of a 7-valent pneumococcal conjugate vaccine (PCV7) aimed at reducing invasive pneumococcal disease in 2000 and a 13-valent pneumococcal conjugate vaccine (PCV13) in 2010, there was an overall reduction in rates of AOM in the United States, especially cases caused by *S. pneumoniae*, including penicillin- and ceftriaxone-resistant strains. However, non-vaccine-type pneumococcal strains and other pathogens continue to cause disease. In addition, AOM is the most common childhood indication for which antimicrobial agents are prescribed, which is of significant

importance in the current era of multidrug-resistant (MDR) and pan-resistant bacteria, in which there is an urgency to reduce unnecessary antimicrobial use. The introduction of an "observation" option for selected children (see "Treatment" section later) has reduced antimicrobial prescriptions for this entity.

Many aspects of AOM parallel those of acute sinusitis, which is not surprising given the proximate locations of these infections and a similar pathogenesis, including preceding viral infection. In older children and adults, the syndrome of middle ear infection, although less common, is still a major precipitant of outpatient office visits during the winter and spring seasons, when viral respiratory infections are common.

Causes

As is the case for acute bacterial sinusitis, viral infections play a pivotal role in the pathogenesis of AOM, preceding bacterial infection in most cases. It is therefore not surprising that viruses have been isolated from the middle ear in some cases. Adenoviruses, rhinoviruses, coronaviruses, respiratory syncytial virus (RSV), influenza virus, and human bocaviruses, among others, have all been recovered from cultures; RSV seems to be particularly associated with AOM.

The microbial cause of the typical case of AOM has been clearly defined by cultures of infected middle ear specimens obtained by direct aspiration from the area. Despite the use of the PCV7 and PCV13, *S. pneumoniae* still accounts for a significant proportion of isolates, likely at least in part because of increasing infections with non-vaccine-type strains. Nontypeable *H. influenzae* and *M. catarrhalis* are other common bacterial pathogens; importantly, both of these can produce β -lactamase, which can affect treatment efficacy. *S. pyogenes* and *S. aureus* have also been implicated in this disease. In patients who have received multiple courses of broad-spectrum antimicrobials for AOM or other infections, have tympanic membrane perforations, or have severe underlying comorbidities or immunosuppression, AOM is more likely to be caused by gram-negative pathogens.

Pathogenesis

The pathogenesis of AOM is similar to that of acute bacterial sinusitis in that a viral upper respiratory tract infection generally precedes or occurs concurrently with most cases of AOM; infection is established when there is subsequent impairment of the normal host defense and drainage mechanisms. The middle ear and eustachian tube, the canal that links the middle ear to the nasopharynx, are lined with ciliated epithelium and mucus-secreting cells. These cells function to prevent infection through the clearance of contaminating infectious agents, thereby inhibiting the passage of bacteria from the nasopharynx to the middle ear cavity. Viral infection impairs this function and can cause mucosal edema, leading to eustachian tube dysfunction and obstruction and development of negative pressure within the middle ear cavity with subsequent entrance of bacteria colonizing the nasopharynx and development of suppurative infection.

In younger children, the eustachian tube is shorter and travels a more direct course from the nasopharynx to the

middle ear, and thus predisposes to easier contamination of the middle ear with nasopharyngeal bacteria. The higher incidence of viral infections in younger children also likely contributes to the increased incidence of AOM in this age group. Suppurative infection can cause fluid buildup in the middle ear that, if prolonged, can affect hearing. In addition, in a small number of cases, infection can spread to adjacent structures.

Clinical manifestations

The diagnosis of AOM is based on signs, symptoms, and direct examination of the tympanic membrane of the ear. Whereas ear pain is the classic symptom of AOM, early signs and symptoms may not be localized, especially in young children. In these cases, fever and irritability may be the only signs of illness. In older children, tugging at the involved ear may be noticed during or at the end of the course of an upper respiratory tract infection. Changes in hearing and, late in the course of infection, drainage of purulent secretions from the ear canal can be associated with perforation of the tympanic membrane. Examination of the tympanic membrane itself classically shows a bulging erythematous or cloudy membrane caused by the presence of fluid, or an effusion, in the middle ear cavity. Pneumatic otoscopy shows decreased mobility of the membrane, consistent with the presence of fluid. In advanced cases, perforation of the membrane with otorrhea, or drainage from the ear, is present.

Complications

Progression of AOM can damage the tympanic membrane, resulting in perforation. Chronic middle ear effusions can also be an issue; in younger children, subsequent hearing loss can have adverse effects on speech development and education that is less of a consequence in adults.

Severe suppurative complications of AOM are rare. Acute mastoiditis, involving extension of the infection to the nearby mastoid air cells of the temporal bone, is an uncommon complication in the antimicrobial era. This can require surgical debridement or resection (mastoidectomy) in addition to antimicrobial therapy to prevent progressive disease. Local extension of infection and spread to the CNS is another serious but uncommon complication.

Laboratory diagnosis

Because of the invasiveness of direct aspiration of fluid from the middle ear and because the predominant pathogens for AOM are known, obtaining specimens for culture before initiating therapy is neither recommended nor necessary in most cases. If an unusual or drug-resistant organism is suspected or the patient fails to respond to empiric antimicrobial therapy, a direct culture of the middle ear fluid by tympanocentesis may be indicated. Culturing the nasopharynx is not helpful in identifying middle ear pathogens; a positive culture for typical otic bacterial pathogens has a low positive predictive value for isolation of the same organism from the middle ear. In addition, viral studies do not provide helpful information in individual cases because it is well known that viral infections precede bacterial AOM, and the presence of virus does not rule out a concomitant bacterial superinfection.

Treatment

The necessity of treating AOM, especially in older children, has been a matter of much debate and illustrates the competing priorities of preventing suppurative complications of the disease and the need for prudent antimicrobial prescribing in an era of MDR bacteria. The natural history of most cases of AOM is resolution of symptoms within a few days to 1 week without antimicrobial therapy. Immediate antimicrobial treatment is recommended in children at high-risk of complications including those younger than 6 months of age, those with severe or persistent symptoms, those with otorrhea, and children 6 to 23 months of age with bilateral nonsevere AOM. Close observation is recommended for others with close follow-up. Tympanostomy tube placement may be considered for recurrent AOM (at least three episodes in 6 months or four episodes in 1 year).

Adults are generally treated at diagnosis because of a lack of data regarding complications. If treatment is indicated, the initial drug of choice in most guidelines is amoxicillin, but in those with risk factors for resistant organisms (β -lactamase-producing *Haemophilus* or *Moraxella*) or those who fail to respond to this initial regimen, an alternative antimicrobial such as amoxicillin-clavulanate should be used. Preventive measures include vaccination for pneumococcus and influenza according to the schedule determined by the Advisory Committee on Immunization Practices (ACIP), encouraging exclusive breast-feeding for the first 6 months, and avoidance of tobacco smoke exposure.

Epiglottitis

Case study

A 4-year-old male patient who recently immigrated to the United States was brought to the pediatrician's office with a 6-hour history of fever and trouble swallowing. Inspiratory stridor was noted by the pediatrician. The patient was immediately taken to the hospital, where, after assurance of airway protection, the epiglottitis was visualized and noted to be red and edematous. He had not received routine childhood vaccinations.

Epidemiology

The epiglottitis is a cartilaginous structure positioned at the anterior aspect of the opening of the trachea whose function is to protect the airway from the aspiration of secretions and food during swallowing. Acute **epiglottitis** (often referred to as *supraglottitis* in adults) can be a rapidly progressive infection of this structure and of adjacent soft tissues in the upper airway, which can result in airway obstruction and respiratory failure, a life-threatening emergency.

Historically, epiglottitis was primarily a disease of children 2 to 7 years of age. However, the epidemiology of epiglottitis changed drastically with the introduction in 1987 of a vaccine for *H. influenzae* type b (Hib), the predominant pathogen causing childhood disease. Since that time, the incidence of epiglottitis in this age group has decreased dramatically, whereas rates in adults have not been significantly affected; older children and adults now form the majority of cases in many studies. Although an uncommon occurrence

in the average clinical practice, the potentially life-threatening nature of this infection warrants familiarity with this syndrome.

Causes

Today, epiglottitis is an uncommon disease. Despite widespread Hib vaccination, Hib is the most common cause of epiglottitis, occurring even in vaccinated individuals. However, the proportion of pathogens other than Hib causing this condition has increased and include, GAS, *S. pneumoniae*, staphylococci (including methicillin-resistant *S. aureus* [MRSA]), nontypeable *Haemophilus* spp., *Klebsiella* spp., and recently SARS-CoV-2. In addition, noninfectious causes, including chemical and thermal irritants, are in the differential diagnosis.

Pathogenesis

The soft tissues of the epiglottis and surrounding structures are susceptible to accumulation of edematous fluid during the inflammatory process incited by infection. Combined with the critical location of the epiglottis at the opening of the trachea, this creates the danger of partial or complete airway obstruction as the disease progresses. It is common, in adults particularly, to involve other structures in the supraglottic region, including the vallecula, arytenoids, and aryepiglottic folds, such that the term *supraglottitis* is often used in adult cases. Epiglottitis has also been linked to sudden infant death syndrome.

Clinical manifestations

In classic childhood cases, the hallmark of epiglottitis is the presence of severe sore throat and pain on swallowing (odynophagia) in the absence of signs of inflammation in the oropharynx on physical examination. The onset of symptoms is abrupt, and in children, a high-grade fever and a toxic appearance are common. Pain with swallowing can be so severe that patients have dysphagia and avoid swallowing secretions, resulting in the classic sign of drooling. Stridor, a high-pitched sound noted during breathing, indicates upper airway obstruction, which is a medical emergency. Adults commonly present in a more indolent fashion and, although sore throat and odynophagia remain common, stridor and respiratory distress are seen much less often. This is likely in part caused by their larger diameter airways, although the risk of airway obstruction still exists, especially with airway manipulation or instrumentation. Underlying patient comorbidities such as diabetes mellitus can increase the risk of severe disease.

The gold standard for diagnosis is direct visualization of the epiglottis with laryngoscopy. Because of the concern for the rapid development of airway compromise and subsequent respiratory failure, this should be done in a setting in which intubation or tracheostomy can be performed immediately to secure the airway, if necessary. Blood cultures and cultures of the epiglottis (only after the airway is secured) should be obtained, and intravenous (IV) antimicrobial therapy should be initiated immediately. A lateral neck x-ray has classically been described as having a thumbprint sign, representing a swollen, edematous epiglottis, but this is not sufficiently sensitive to rule out disease in its absence. The differential

diagnosis for epiglottitis in children includes other causes of airway obstruction, including croup (laryngotracheitis), bacterial tracheitis, and retropharyngeal abscess.

Croup is the most common cause of infectious airway obstruction in young children 6 months to 3 years of age; most cases are caused by viral infections, usually parainfluenza virus type 1 or 3. Swelling of the subglottic structures, larynx and trachea, is responsible for the symptoms. Although epiglottitis tends to have an abrupt onset of symptoms, croup tends to have a more insidious onset, with a viral prodrome of rhinorrhea, congestion, and a harsh barking cough. Like epiglottitis, patients may also present with stridor if upper airway obstruction is present.

Bacterial tracheitis, like epiglottitis, is uncommon and occurs primarily in young children. The most likely causative organisms include *S. aureus*, *S. pneumoniae* and other streptococci, and *M. catarrhalis*. Clinically, the disease starts with a viral prodrome similar to croup and then progresses to a more toxic appearance, with fever and respiratory distress, resembling epiglottitis. These patients develop subglottic edema and thick tracheal secretions.

Complications

The most important complication of epiglottitis is respiratory compromise progressing to respiratory failure because of airway obstruction from edema of the supraglottic structures. Other potential complications include the development of epiglottic abscess, necrotizing epiglottitis, pneumonia, and in patients who are bacteremic, meningitis and septic shock.

Laboratory diagnosis

Direct swab cultures from the area of the epiglottis are useful for establishing the causative diagnosis, but they should be taken only when the airway is secure. A direct smear of epiglottic exudates for microscopic examination may reveal numerous WBCs and pleomorphic, gram-negative bacilli characteristic of *H. influenzae* (Fig. 32.4). Blood cultures should be performed in all patients in whom epiglottitis is suspected. An enriched medium such as chocolate agar in an environment with an increased concentration of carbon dioxide (5% to 10%) is preferred when trying to isolate *Haemophilus* spp.

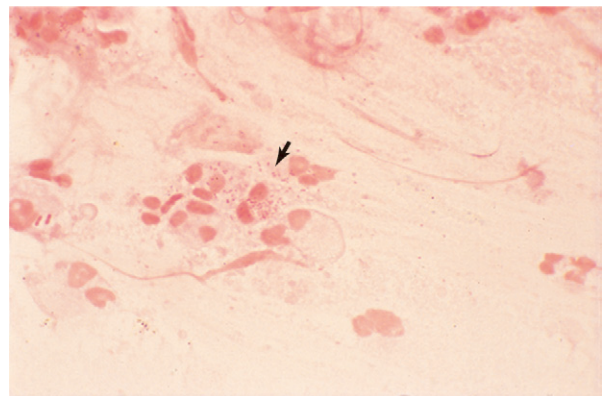


Fig. 32.4 Gram-stained smear of sputum-exudate with gram-negative coccobacilli suggestive of *Haemophilus* spp. (arrow).

Treatment

IV antimicrobials, such as third-generation cephalosporins (e.g., cefotaxime, ceftriaxone) or ampicillin-sulbactam, should be administered. Coverage for MRSA should be added when risk factors exist. If immediate airway securement is not required at the time of initial evaluation, patients should be monitored closely for signs and symptoms of respiratory distress, which might require subsequent urgent intubation or tracheostomy. IV corticosteroids have been given, theoretically to help reduce inflammation and swelling of the airway, although their efficacy has not been proven in research studies.

Case check 32.4

Epiglottitis, especially in children, can be a life-threatening emergency because of the risk of airway compromise. Any attempts to visualize or culture the supraglottic region should be performed only in a controlled setting where airway protection can be assured. Historically, *Haemophilus influenzae* type b (Hib) was responsible for the majority of cases. The rates of infection with this particular organism have drastically decreased since the introduction of childhood vaccination for Hib, but cases still occur, especially in unimmunized populations.

Pertussis

Epidemiology

Pertussis, commonly referred to as *whooping cough*, is a highly communicable respiratory illness in susceptible patient populations that is transmitted from person to person via aerosolized droplets. This infection usually occurs in infants and young children, and serious complications are seen more often in this age group. Following the introduction of the first pertussis vaccine in the 1940s, the incidence of pertussis significantly decreased. However, in the United States the disease incidence began increasing in 2010 reaching a 50-year high of 48,277 cases in 2012, after which case numbers began decreasing. During this surge, large outbreaks in several states with notable infant mortality and increased cases in the adolescent and adult population were reported. In 2022, the number of provisional cases for 2021 was 1609. Older adult patients can exhibit milder manifestations of the illness that can go undiagnosed and allow them to serve as reservoirs for transmission of the infection to susceptible children and others.

Causes

Bordetella pertussis and *Bordetella parapertussis* are the pathogens usually associated with pertussis; *Bordetella bronchiseptica* and *Bordetella holmesii* have been associated with a similar clinical syndrome. An important differential diagnosis in patients presenting with a pertussis-like syndrome is adenovirus infection (serotypes 1, 2, 3, and 5).

Pathogenesis

Although the pathogenesis of pertussis is incompletely understood, numerous studies suggest a significant role for pertussis toxin (PT), which interrupts the transduction of signals from cell surface receptors to intracellular systems. PT is included in all currently licensed acellular pertussis vaccines.

Another toxin is adenylate cyclase toxin that enters target cells and increases intracellular cyclic adenosine monophosphate (cAMP) levels, causing cell damage or cell death. Factors produced by these bacteria have been implicated in several stages in the disease process, including paralysis of the cilia of lower respiratory tract cells, damage to tracheal epithelial cells, impairment of host immunity, and induction of a systemic response to pertussis. During the paroxysmal phase of the illness, when patients develop the characteristic whooping cough, the clinical signs and symptoms are attributed to toxins elaborated by the organisms. It is apparent that pertussis is not a disease caused by the effects of a single toxin, as is the case in some other bacterial infections. Rather, the clinical syndrome is probably the manifestation of the cumulative effects of several toxins produced by the pathogen and the related host inflammatory response.

Clinical manifestations

In the initial catarrhal phase of the illness, the symptoms of pertussis are similar to those of a viral upper respiratory tract infection, with mild cough, conjunctival injection, and coryza (nasal congestion and discharge), making the differential diagnosis difficult for the first 1 to 2 weeks. Fever is uncommon or low grade throughout the course of the illness unless a secondary bacterial infection has occurred. The 2- to 4-week paroxysmal phase follows and is typically manifested by exhausting paroxysms of coughing, often with multiple coughs during one expiratory cycle and a characteristic whooping sound at the conclusion caused by forceful inspiration through a narrowed airway. Infants may develop apneic episodes rather than a whooping sound. Posttussive vomiting or syncope is not uncommon because of the severity of coughing. Symptoms are often worse at night. The final convalescent stage may last for several more weeks and comprises a chronic cough.

Complications

Severe infections requiring hospitalization occur most commonly in infants, especially those younger than 2 months of age. Older adults have increased rates of hospitalization compared with other age groups as well. The most common complication is pneumonia, which is seen in up to 20% of cases. This is usually caused by secondary infections with other bacteria, although *B. pertussis* infection itself can cause lower respiratory tract infection. Encephalopathy and seizures are seen in a small fraction of cases but can have serious consequences. In a minority of cases, pertussis can result in death, overwhelmingly as a consequence of pneumonia in young, unvaccinated children or older adult patients with underlying comorbid conditions, such as chronic pulmonary disease.

Many of the complications associated with pertussis are a result of the severe and forceful coughing episodes that occur, including pneumothorax, subconjunctival (and other superficial) hemorrhages, epistaxis (nosebleed), rupture of the diaphragm, umbilical and inguinal hernias, and rectal prolapse. This infection has been implicated as a cause of bronchiectasis later in life.

Laboratory diagnosis

A complete blood count (CBC) may show lymphocytosis, which is a reaction to PT, and has been associated with poor

outcomes in infected infants. Isolation of *B. pertussis* in culture from nasopharyngeal secretions is the historic gold standard for diagnosis and exhibits high specificity. However, culture recovery of *B. pertussis* can be difficult; sensitivity depends on the timing of the culture relative to disease onset, administration of antimicrobials before culture, and proper specimen collection and processing. Specimens should be collected from the posterior nasopharynx early in the course of disease (preferably within the first 2 weeks of onset of cough) and should be plated directly onto selective media, such as Bordet-Gengou or Regan-Lowe (RL) medium for optimal recovery. If direct inoculation is not possible, a transport medium such as RL transport medium should be used.

Because of the time delay in obtaining culture results and the inherent difficulties in culture recovery, culture has largely been superseded in clinical practice by PCR assay. PCR has the advantage of increased sensitivity compared with culture when used in the proper clinical situation, including in patients in whom antimicrobial therapy has already been initiated, which may result in false-negative culture results. As with culture, specimen testing is best done during the first few weeks of illness. PCR testing should be used only for patients with signs and symptoms suggestive of pertussis and should not be used for testing of contacts. The development of multiplex PCR has allowed detection of multiple related pathogens with one test; as such, *B. pertussis* testing may occur in the context of testing for multiple respiratory pathogens at once. Direct fluorescent antibody (DFA) testing has been used as a complementary method but is not recommended because of poor sensitivity and specificity.

Serologic testing may be helpful 2 to 12 weeks after the onset of cough, when culture and PCR results are more likely to be negative, and it is more useful for outbreak investigation. Several EIAs and bead-based assays are available to detect IgG or IgA antibody response to many different antigens. The recommended diagnostic assay is quantifying anti-PT antibody. Serologic measurements should be paired and obtained 2 to 4 weeks apart to assess the patient for a fourfold increase in titer to establish the diagnosis.

Treatment

Antimicrobial therapy often does little to help with symptom resolution unless given early in the course of the illness, but therapy plays an important public health role in minimizing the spread of infection. The macrolide class of antibiotics, including azithromycin and clarithromycin, is the therapy of choice; trimethoprim-sulfamethoxazole is an alternative. Droplet isolation precautions should be applied for hospitalized patients until 5 days after initiation of appropriate antimicrobial therapy. Given the often very high secondary attack rates, the same agents are also recommended as chemoprophylaxis for those who have had close contact with a patient with pertussis in the first few weeks of disease, regardless of pertussis vaccination status.

Vaccination against pertussis has been shown to prevent disease in many recipients and decrease the severity of illness in infected young infants whose mothers were vaccinated during the third trimester of pregnancy. Rates of vaccination of infants and young children with DTaP (diphtheria toxoid, tetanus toxoid, and acellular pertussis) vaccine remain high, but adolescents and adults are at risk because of their

waning immunity. Starting in 2005, the ACIP recommended the use of a DTaP vaccine to boost immunity in adolescents and adults including all pregnant women between 27 and 36 weeks' gestation; unfortunately, the vaccination rate among adults 19 years of age and older was only around 23% in 2015 and 32% in 2017. In 2019, among the 4674 patients 6 months to 6 years of age diagnosed with pertussis, only 37% had received three or more doses of DTaP. In 2020, the Centers for Disease Control and Prevention reported a substantial decline in DTaP vaccination in children during the COVID-19 pandemic—15.7% among children younger than 24 months and 60.3% among children aged 2 years to 8 years.

Lower respiratory tract infections

Infections in the lower respiratory tract usually occur when infecting organisms reach the lower airways or pulmonary parenchyma via bypassing the mechanical and other nonspecific barriers of the upper respiratory tract. Infections may result from inhalation of infectious aerosols, aspiration of oral or gastric contents, or hematogenous spread.

A series of host defenses must be overcome before a potential pathogen can establish infection in the lower respiratory tract. The sequence of events is somewhat different for respiratory viruses than for bacterial pathogens. The progression of viral pathogens from the upper to the lower respiratory tract is a process that involves spread among adjacent cells and distant inoculation of susceptible cells by aspiration of infectious secretions and, to a lesser extent, by hematogenous transmission of the virus. Lung infections by bacterial pathogens usually occur via direct inoculation of organisms through aspiration from the upper respiratory tract. [Table 32.2](#) summarizes clinical syndromes encountered in the lower respiratory tract and their associated causative agents.

Bronchitis and bronchiolitis

Epidemiology

Acute **bronchitis** is an infection and inflammation of the bronchi without involvement of the lung parenchyma (pneumonia). Bronchitis can be viewed as the lower respiratory tract extension of many of the same viral infections that cause seasonal upper respiratory tract infections; in many cases, these syndromes represent a continuum of the same infection. The peak season for acute bronchitis is the winter months, which correlates with the period of peak incidence of these viral respiratory tract infections. Acute **bronchiolitis**, an infection and inflammation of the smaller bronchioles, is the most common lower respiratory tract infection in infants and is most often seen in children between 3 and 6 months of age.

Causes

Any of the respiratory viruses that cause upper respiratory tract infection can cause cough as a manifestation of acute bronchitis. During seasons when influenza virus is epidemic in the community, this respiratory pathogen is the most common cause of acute bronchitis in the general population. During nonepidemic periods, other respiratory viral

Table 32.2 Lower respiratory tract infections

Clinical syndrome	Causative agents	Specimen collection	Other
Bronchitis, bronchiolitis	<u>Most common</u> RSV Influenza virus <u>Less common</u> <i>Mycoplasma pneumoniae</i> <i>Chlamydia pneumoniae</i> <i>Bordetella pertussis</i>	Nasopharyngeal or lower respiratory tract sample if influenza or pertussis is suspected. RSV in select cases Diagnostic testing not indicated in uncomplicated cases	Viral PCR test Viral direct antigen detection Viral culture
Community-acquired pneumonia	Children: <u>Most common</u> RSV Influenza virus A, B Parainfluenza virus 1, 2, 3 Adenovirus <i>M. pneumoniae</i> <u>Less common</u> <i>Streptococcus pneumoniae</i> <i>Staphylococcus aureus</i> <i>Haemophilus influenzae</i> Group B <i>Streptococcus</i> (neonates) Adults: <u>Most common</u> <i>S. pneumoniae</i> <u>Less common</u> <i>M. pneumoniae</i> <i>H. influenzae</i> <i>C. pneumoniae</i> Respiratory viruses <i>Legionella pneumophila</i>	Deep expectorated sputum. Avoid contamination with oropharyngeal biota; specimen collection via fiber-optic bronchoscopy or open lung biopsy may be indicated in refractory or severe cases	Viral direct antigen detection Viral and bacterial PCR assays Bacterial culture <i>S. pneumoniae</i> and <i>Legionella</i> serogroup 1 urinary antigens <i>Legionella</i> cultures on selective media (BCYE) Assess for SARS-CoV-2 infection
Hospital-acquired and ventilator-associated pneumonias	Gram-negative bacilli <i>S. aureus</i> <i>S. pneumoniae</i> <i>H. influenzae</i> <i>L. pneumophila</i>	Deep expectorated sputum and endotracheal aspirate (avoid contamination with oropharyngeal biota); specimen collection via fiber-optic bronchoscopy or open lung biopsy may be indicated in some cases but is not routinely recommended	Bacterial culture <i>Legionella</i> serogroup 1 urinary antigens <i>Legionella</i> cultures on selective media (BCYE) Influenza virus and SARS-CoV-2 PCR tests during community epidemics and institutional outbreaks
Aspiration pneumonia	Mixed aerobes and sometimes anaerobes	Generally treated empirically. Respiratory sampling same as other pneumonias. Pleural fluid cultures may be useful with empyema	Bacterial culture
Chronic pneumonia or lung granulomatous disease	<i>Mycobacterium tuberculosis</i> Nontuberculous mycobacteria (including <i>Mycobacterium avium</i> complex, <i>Mycobacterium chelonae-abscessus</i> complex, and <i>Mycobacterium fortuitum</i> group) <i>Blastomyces dermatitidis</i> <i>Histoplasma capsulatum</i> <i>Coccidioides immitis</i>	Deep, expectorated sputum; induced sputum, bronchoscopy or trans-bronchial or open lung biopsy may be required to identify pathogen Deep expectorated sputum; induced sputum, bronchoscopy or trans-bronchial or open lung biopsy may be required to identify pathogen Lower respiratory tract cultures or tissue. If extrapulmonary disease present, other tissue can be obtained for culture Lower respiratory tract cultures or tissue. If extrapulmonary disease present, other tissue can be obtained for culture	• Acid-fast bacilli stain • Mycobacterial culture • Nucleic acid amplification assays for sputum • Acid-fast bacilli stain • Mycobacterial culture • Fungal cultures • Histopathology and fungal stains of tissue such as GMS, calcofluor white, and PAS • Urinary and serum antigen test • Fungal culture • Histopathology and fungal stains of tissue • Serology

Continued

Table 32.2 Lower respiratory tract infections—Cont'd

Clinical syndrome	Causative agents	Specimen collection	Other
	<i>Cryptococcus neoformans</i> and <i>Cryptococcus gattii</i>	Lower respiratory tract or cerebrospinal fluid cultures	• Serum, cerebrospinal fluid, or urine cryptococcal antigen test • Fungal culture • Histopathology and fungal stains of tissue
	<i>Aspergillus</i> spp.	Lower respiratory tract cultures or tissue. If extrapulmonary disease present, other tissue can be obtained for culture	• Fungal culture • Histopathology and fungal stains of tissue • Galactomannan assay
	Zygomycetes	Lower respiratory tract cultures or tissue. If extrapulmonary disease present, other tissue can be obtained for culture	• Fungal culture • Histopathology and fungal stains of tissue
	<i>Pneumocystis jiroveci</i>	Induced sputum or bronchoalveolar lavage specimen	• GMS stain of tissue • Direct fluorescent antibody stain • PCR
Empyema	<u>Community acquired</u> <i>S. pneumoniae</i> <i>S. aureus</i> <i>Streptococcus pyogenes</i> <i>Streptococcus anginosus</i> Anaerobes <i>M. tuberculosis</i> <u>Hospital acquired</u> Gram-negative bacilli Anaerobes	Pleural fluid should be aspirated directly into a sterile syringe, with excess air expelled immediately from syringe or aseptically inserted in prereduced transport medium	Bacterial culture Aliquots of specimen should be distributed to hematology and chemistry laboratories for other studies

BCYE, Buffered charcoal-yeast extract agar with 0.1% α -ketoglutaric acid; GMS, Gomori methenamine silver; PAS, periodic acid-Schiff; PCR, polymerase chain reaction; RSV, respiratory syncytial virus.

pathogens such as rhinovirus, coronavirus, human metapneumovirus (hMPV), and parainfluenza virus are often identified. In populations of young military recruits, adenovirus infections were the primary cause of acute bronchitis before the use of adenovirus vaccine. Nonviral respiratory tract pathogens, including *M. pneumoniae*, *Chlamydia pneumoniae*, and *B. pertussis*, can also produce acute bronchitis. The clinical presentation of these infections may be indistinguishable from that of acute bronchitis caused by viral pathogens.

Bronchiolitis in infants is predominantly caused by RSV, which accounts for 41% to 83% of cases, primarily occurring in yearly epidemics during the winter months. It is the most common cause of serious lower respiratory tract infections in children. Rhinovirus is also identified in a significant number of cases. In up to 30% of bronchiolitis infections, more than one viral pathogen may be isolated; the clinical implications of this finding are unclear.

Pathogenesis

As noted, evidence almost always exists of an antecedent or coexistent upper respiratory tract infection in patients with acute bronchitis. The spread of these upper respiratory tract infections to the lower airways, manifested as acute bronchitis, represents infection and damage of respiratory epithelial cells by the same pathogens. The extent of destruction of the respiratory epithelium differs with the pathogen causing the illness. Viruses such as influenza virus and adenovirus are highly cytopathic and cause significant epithelial cell destruction; other viral infections such as rhinovirus infections cause

epithelial cell dysfunction without producing much cell destruction.

The inflammatory response, necrotic debris from epithelial cell destruction, bronchospasm, and edema of the lower respiratory tract also contribute to the airway abnormalities and symptoms in these infections. This potential for airway obstruction, air trapping, and increased airway resistance is especially important in bronchiolitis in infants. The resulting obliteration of the lumen of small airways appears to be the primary pathogenic mechanism causing lower respiratory tract symptoms. T-cell response is critical in controlling RSV infection such that immunocompromised infants with deficient cell-mediated immunity may have a more severe infection.

Clinical manifestations

Patients with acute bronchitis often begin their illness with a syndrome typical of a viral upper respiratory tract infection, including rhinorrhea and nasal congestion. Systemic symptoms such as diffuse myalgias, low-grade fevers, and fatigue associated with viremic illnesses may also be seen early in the course of infection. The hallmark of acute bronchitis is a cough that can persist for as long as 6 weeks. The amount of sputum produced with coughing differs; the color of the sputum produced does not reliably distinguish bacterial from viral bronchitis.

In addition to signs of an upper respiratory tract infection, acute bronchiolitis patients present with signs of lower respiratory tract airway obstruction, such as expiratory wheezing

and respiratory distress. Young infants may also experience apnea. A persistent cough is common. The median duration of symptoms is 2 weeks, but in some cases can be more prolonged, especially in immunocompromised individuals.

Complications

A complication in some patients with acute viral bronchitis is the development of a secondary bacterial infection, either bronchitis or pneumonia. These secondary bacterial infections of the lower respiratory tract are usually caused by the common pathogens found in community-acquired pneumonia (CAP), such as *S. pneumoniae* and *H. influenzae*, although other bacterial pathogens (e.g., *M. catarrhalis*, gram-negative bacilli) may be involved in select patient populations (e.g., patients with underlying respiratory diseases, hospitalized patients).

Severe bronchiolitis is more common in younger, especially premature, infants. Infants with chronic cardiac and pulmonary disease are also at increased risk. As is the case for bronchitis, secondary bacterial infections can also occur in a subset of bronchiolitis patients. Concurrent otitis media may also be present. Although bronchiolitis causes significant morbidity in many cases, overall mortality has remained low.

There is also speculation that certain viruses that cause acute bronchitis can cause bronchiectasis. This is an abnormality in which inadequate drainage of respiratory secretions results from airway wall destruction, dilation, and scarring associated with recurrent lower respiratory tract infections. Considering the cytopathic nature of some viral infections (e.g., influenza, adenovirus) that can cause acute bronchitis, the suggested association between these pathogens and long-term development of bronchiectasis is a reasonable assumption.

Laboratory diagnosis

Because most bronchitis infections are caused by viruses with no available directed therapy, diagnostic testing was not previously recommended in uncomplicated cases that follow the expected, self-limiting course unless there was another indication for testing. During the COVID-19 pandemic, however, many of these patients received viral diagnostic testing for epidemiologic purposes or to complement therapeutic decision making. In patients in whom there are signs of secondary bacterial bronchitis, pertussis is suspected, or pneumonia is a concern, diagnostic testing may be useful in guiding therapy. In cases of suspected influenza virus infection of the lower respiratory tract, identification of the virus in respiratory tract secretions may be useful in guiding early antiviral therapy. The test of choice for most viral pathogens currently is a PCR assay because viral cultures generally have low sensitivity.

For patients with RSV, routine diagnostic testing is not recommended; diagnosis should be based on history and physical examination. RSV testing by viral antigen or PCR assay is available if needed to aid in patient cohorting for infection control purposes during outbreaks. In patients with bronchiolitis, chest x-rays do not usually show typical signs of pneumonia and can range in appearance from a normal chest x-ray to one showing peribronchial thickening, hyperinflation, or patchy consolidation. In most typical clinical cases, routine chest radiography is therefore not recommended because it does not change treatment.

Treatment

Acute bronchitis is caused by viral infection more than 90% of the time and therefore should not be treated with antimicrobial therapy in most otherwise healthy individuals without concurrent pneumonia. Antimicrobial therapy has not been shown by systematic review to be beneficial in acute uncomplicated bronchitis, and there was a trend toward increased adverse events in the treatment group. Despite this, 70% of outpatient visits for acute bronchitis result in an antibacterial prescription. Anti-influenza therapy ideally should be started within 48 hours of onset of symptoms for it to have a significant effect on clinical outcome, but in hospitalized patients or those at high risk for complicated or severe disease, antiviral therapy is recommended even after this time. In secondary bacterial bronchitis and/or pneumonia, antimicrobial treatment regimens can be designed using the same approach used for acute bacterial pneumonia. Pertussis can be treated with macrolide therapy (see the "Pertussis" section earlier in this chapter).

Treatment of RSV infection is generally supportive, including maintenance of hydration and supplemental oxygen when necessary. Ribavirin resistance has not been noted. Studies of inhaled bronchodilators and nebulized hypertonic saline have not shown consistent results. Palivizumab, a monoclonal antibody, can be used for prophylaxis during the RSV season in children at highest risk for bronchiolitis complications.

Influenza

Epidemiology

Seasonal flu activity begins in late fall and continues into the early spring. Cases are more frequent in northern climates. During pandemics, however, cases can occur outside the typical season. Between 2010 and 2020, the Centers for Disease Control and Prevention (CDC) estimates 9 million to 41 million influenza cases occurred, resulting in 710,000 hospitalizations and 12,000 to 50,000 deaths annually.

Causes

Influenza is an acute illness caused by influenza A virus or influenza B virus. Influenza A viruses are categorized into subtypes based on two surface antigens: hemagglutinin (H) and neuraminidase (N). Influenza B viruses are categorized into lineages. Influenza A is responsible for pandemics.

Pathogenesis

Human influenza viruses continuously undergo a process of antigenic drift, whereby amino acid substitutions allow the virus to evade preexisting host immunity. This *antigenic drift* is responsible for annual worldwide outbreaks and occurs more commonly in influenza A viruses. Periodically, two strains of influenza A virus will combine genetic material, often in animals, and result in the generation of a new reassorted virus to which there is often little population (herd) immunity. This process of genetic reassortment is termed *antigenic shift*. If the new virus contains virulence factors that include efficient person-to-person transmission, a worldwide influenza outbreak, or pandemic, can occur, with the potential for increased morbidity and mortality. The influenza pandemics in 1957 and

1968 were caused by genetic reassortment between human influenza viruses and influenza viruses from birds. The novel H1N1 influenza pandemic in 2009 was caused by the reassortment of genetic elements of human, swine, and avian strains.

Clinical manifestations

Respiratory tract infection with systemic symptoms of fever, myalgias, and fatigue is the most classic manifestation of influenza, but some patients may present only with rhinorrhea and cough, similar to other upper respiratory tract viral infections. Most cases involve the upper respiratory tract, but pneumonia can occur as well, either secondary to direct effects of the virus itself or because of bacterial superinfection.

Complications

Young children, older adults, pregnant women, and patients with underlying comorbidities, including heart and lung disease, diabetes, renal disease, morbid obesity, and immunosuppression, are at increased risk for severe disease, which in some groups includes an increased risk of developing primary influenza pneumonia. Hospitalization rates are increased in young children and older adults, and mortality is generally highest in older adults. Influenza infection also predisposes patients to develop secondary bacterial pneumonia; bacterial pathogens commonly seen in this setting include *S. pneumoniae*, *S. aureus* (including community-associated strains of MRSA), and *H. influenzae*.

Diagnosis

Previously, during an influenza outbreak, acute febrile respiratory illnesses could be diagnosed as influenza with a relatively high degree of certainty by clinical criteria alone, and testing was not always necessary. However, during the COVID-19 pandemic, influenza cases could not be reliably differentiated from infections caused by SARS-CoV-2 or other respiratory viruses on clinical grounds, and there is increased emphasis on testing for treatment, infection control and prevention, and epidemiologic surveillance purposes.

Diagnosis is accomplished by the detection of virus, viral antigen, or viral nucleic acid in nasopharyngeal swabs, mid-turbinate nasal swabs, nasal washes or aspirates, combined nasal and throat swabs, sputum, or bronchoalveolar lavage specimens, depending on the assay. Isolation of the virus in culture is highly sensitive and specific but may take several days after inoculation and therefore is not commonly performed for clinical care. Antigen detection can be performed quickly with high specificity but is insufficiently sensitive to rule out influenza. Molecular assays such as reverse-transcription polymerase chain reaction (RT-PCR) are now the standard of care with high sensitivity and specificity, the ability to distinguish among influenza A subtypes, and a turnaround time in hours. Multiplex PCR assays that can also detect SARS-CoV-2 are increasingly being used. The diagnosis can also be established retrospectively by serologic methods; a fourfold or greater increase in antibody titers between serum specimens obtained during acute illness and convalescence approximately 2 to 4 weeks later is considered diagnostic but will not inform timely clinical management.

Treatment

The neuraminidase inhibitors oseltamivir, zanamivir, and peramivir and the cap-dependent endonuclease inhibitor baloxavir are recommended for the treatment of influenza depending on the age, clinical characteristics, and disease severity of the individual. Adamantane-based M2 inhibitors, such as amantadine, are no longer indicated for influenza treatment because of widespread resistance. In healthy patients, antiviral therapy should be started within 48 hours of symptom onset to decrease the duration of symptoms and prevent complications such as pneumonia; therapy can also be considered for those with high-risk close contacts. In patients who require hospitalization, those with severe or progressive illness, or those at risk of significant complications, antiviral therapy is indicated regardless of illness duration.

The best way to prevent influenza is by annual vaccination starting in the fall of each year. Vaccines are manufactured annually based on global virus surveillance data to allow inclusion in the vaccine of influenza A and influenza B strains that are most representative of those strains circulating worldwide. Influenza vaccination is recommended for all persons at least 6 months of age who do not have contraindications. Antiviral therapy can also be used for chemoprophylaxis before or after exposure to prevent development of disease for high-risk patients, such as those who have vaccine contraindications or expected poor vaccine efficacy (i.e., caused by severe immunosuppression). Antiviral chemoprophylaxis is also indicated for patients and staff as part of the management of institutional outbreaks.

Emerging viral respiratory tract infections: coronaviruses

A number of emerging viral respiratory tract infections have been seen in the past few decades including avian influenza strains, pandemic H1N1 influenza A, hMPV, and reemerging adenoviruses. However, novel human coronaviruses have arguably had the most wide-reaching global consequences and will be reviewed here.

Severe acute respiratory syndrome and Middle East respiratory syndrome

SARS, a viral pneumonia, is caused by SARS-CoV that was first recognized in southern China in late 2002. This virus was believed to be of animal origin and was identified in civets and bats. The CDC estimates that 8098 infections and 774 deaths were attributed to this outbreak until it was contained in China in 2004. Although the initial cases were identified in China and Hong Kong, the disease spread rapidly, and cases were seen in five continents. Human-to-human transmission (direct contact with respiratory secretions and spread via respiratory droplets) was the primary mode of spread, and it was commonly seen in health care facilities. Airborne transmission was also postulated in the case of superspreading events on commercial airplanes.

Middle East respiratory syndrome (MERS) is also a viral pneumonia caused by a coronavirus. MERS was first recognized in Saudi Arabia in 2012, and cases have since been reported in the United States, Europe, and Asia, all of which were associated with travel to the Arabian Peninsula or

contact with a patient with MERS coronavirus (MERS-CoV). Camels appear to serve as a reservoir of MERS-CoV, but most cases have resulted from person-to-person transmission. Differences between MERS and SARS are that MERS-CoV does not spread as easily among humans, MERS pneumonia commonly occurs in patients with preexisting chronic illnesses, and it has a higher mortality rate than SARS.

Recommended viral diagnostic studies for SARS and MERS include nucleic acid amplification tests (NAATs) such as RT-PCR. Diagnosis requires multiple different clinical specimens for confirmation; lower respiratory, nasopharyngeal, serum, and stool specimens are usually used depending on the infection. Because viral shedding peaks around day 5 to day 10, those with initial negative test results may be retested later in the course if an alternative diagnosis has not been found and a high suspicion for SARS or MERS remains. A biosafety level 3 laboratory is required for handling specimens to prevent infection in laboratory personnel. Currently no proven effective antiviral medication exists; supportive care with appropriate isolation to prevent transmission is critically important.

COVID-19

In December 2019, a cluster of viral pneumonia cases was reported in Wuhan, China. A novel human coronavirus was quickly identified as the cause, later to be named SARS-CoV-2; the disease was termed *coronavirus disease 2019* (COVID-19). The infection rapidly became endemic in China, and in March 2020, the World Health Organization (WHO) declared a global pandemic. As of July 2022, over 570 million cases and more than 6.3 million deaths have been reported worldwide. The virus undergoes mutations producing new variants that cause a surge in cases. Our understanding of the epidemiology, clinical manifestations, diagnosis, treatment, and prevention of this disease continues to rapidly evolve.

Like SARS-CoV and MERS-CoV, SARS-CoV-2 is a betacoronavirus. Transmission is thought to be primarily through exposure to large respiratory droplets, but airborne transmission has also been recognized; contact with contaminated objects and secretions/excretions is thought to play a lesser role. Unlike influenza, it was subsequently realized that many infected individuals are asymptomatic, and both asymptomatic and presymptomatic transmission are significant contributors to the spread of infection, making containment particularly challenging.

The clinical presentation includes a spectrum from asymptomatic infection to severe pneumonia with respiratory failure, acute respiratory distress syndrome (ARDS), and multisystem organ failure. Children of all ages can be infected but generally seem to have milder symptoms than adults, although severe disease does occur. Risk factors for severe disease include older age; obesity; diabetes; chronic lung, heart, or kidney disease; and immunocompromised state. For many who develop symptoms, the disease has a characteristic clinical decline in the second week after onset. In addition to respiratory symptoms such as cough and shortness of breath, typical flulike symptoms (fever, headache, muscle aches, fatigue) are common as are gastrointestinal (GI) manifestations including diarrhea. An altered sense of taste or smell may also be present. COVID-19 has also been associated with a coagulopathy.

Complications of COVID-19 infection are still incompletely understood. Respiratory failure and ARDS are the most common end organ complications, but many organ systems can be affected including renal, hepatic, cardiac, and neurologic. There is a growing body of literature that a subset of infected patients has prolonged symptomatology, even after initial recovery, referred to as *long COVID*. Multisystem inflammatory syndrome in children (MIS-C) is a constellation of symptoms that can include fever, GI complaints, rash including mucocutaneous involvement, dyspnea, headache, and lethargy among others. Hypotension, myocardial involvement, and elevated inflammatory markers are common. It is thought that the syndrome results from immune dysregulation (cytokine storm) that develops after acute infection with SARS-CoV-2 as many children have negative molecular testing but positive antibodies, suggesting recent infection. This syndrome can occur in older adult patients as well, usually in young to middle-age adults.

Accurate diagnostics for SARS-CoV-2 are critical as COVID-19 cannot reliably be differentiated from other infections based on clinical manifestations alone, and test results affect treatment, public health, and infection prevention and control decisions. Testing is generally recommended for all symptomatic individuals as well as for some asymptomatic individuals in cases in which exposure has occurred or results will affect personal protective equipment usage and isolation decisions. NAATs have become the standard assay. These are generally performed on a nasopharyngeal, midturbinate, or anterior nares swab sample; saliva testing is also available. Sensitivity is high, especially for laboratory-based tests, but false-negative results do occur. NAATs have the disadvantages of higher cost, longer turnaround times, and persistent detection of viral RNA when ongoing transmissibility is unlikely. Repeat testing is not recommended within 90 days of a prior positive NAAT. Viral antigen assays that can be performed at the point of care are easy to use and provide quick results. Sensitivity is generally higher in symptomatic patients at the peak of infection, but lower than for NAATs, especially in asymptomatic individuals.

Multiple commercial antibody tests are now available to detect antibodies to either the spike or nucleocapsid proteins of SARS-CoV-2 using varied technologies; performance may vary among assays. Antibodies are generally not present before 2 weeks after symptom onset and are not recommended for ruling out acute infection. Antibody testing may be helpful in symptomatic patients with a high clinical suspicion and repeatedly negative NAATs, ideally 3 to 4 weeks after symptom onset. Antibody testing also has a role in diagnosing MIS-C and epidemiologic seroprevalence studies. It should be remembered that vaccination against SARS-CoV-2 can also result in detectable antibodies against the spike protein. Immune protection cannot be inferred based on current antibody testing data.

Several COVID-19 therapies and treatment guidelines are now available. Therapies include the antiviral agents ritonavir-boosted nirmatrelvir (Paxlovid) and immunomodulators dexamethasone, baricitinib, and tocilizumab (a monoclonal antibody against the IL-6 receptor). Supportive therapy includes high-flow nasal canula oxygen, mechanical ventilation, and extracorporeal mechanical membrane oxygenation.

Prevention of SARS-CoV-2 infection with vaccination is thought by many to be critical to ending the pandemic.

Currently, the U.S. Food and Drug Administration (FDA) has granted Emergency Use Authorization for three vaccines in the United States, two of which use a novel messenger RNA platform that has been studied for decades but was never previously FDA approved. Current available data indicate excellent efficacy with minimal reports of serious adverse events; postapproval safety monitoring is ongoing. Further surveillance of vaccine efficacy in the face of newly discovered viral variants will also be important.

Acute pneumonia

The distinction between acute bronchitis and acute **pneumonia** may be subtle. The distinction depends on the extent of involvement of the lower respiratory tract with the infectious process. Patients who have bronchitis do not exhibit the physical, radiographic, and pathologic findings of pulmonary parenchymal involvement (fluid accumulation in the alveoli) outside the airways. This type of lung tissue involvement with the infectious process defines pneumonia.

Pneumonia can be subdivided into diagnostic categories based on the clinical setting, presentation of the illness, exposure to specific pathogens, and age and type of host infected. The importance of using such a strategy to make a presumptive determination of the infectious cause of pneumonia is evident when one considers the many possible pathogens that can cause this type of infection; Table 32.3 lists the most common causative agents of lower respiratory tract infections, stratified by age group. It is important to focus the clinical diagnosis on a subgroup of likely pathogens to allow the initiation of reasonable empiric therapy while awaiting a specific causative diagnosis by the microbiology laboratory. The highest incidence of CAP and hospital-acquired pneumonia (HAP) occurs in very young and very old patients. However, the types of causative agents most likely to cause pulmonary infections in these two groups are different. In infants and children, respiratory viruses cause most pneumonias; in older adults, bacterial pathogens are more likely to be implicated.

Community-acquired pneumonia

Epidemiology

CAP is one of the most common infections encountered in clinical practice and the leading cause of death from infection in persons older than 65 years of age. The onset of symptoms is in the community or within the first 2 days after hospital admission. Vaccination against *S. pneumoniae*, the most common bacterial pathogen in children and adults, has resulted in a decrease in invasive pneumococcal disease (e.g., bacteremia, meningitis) from vaccine serotypes as well as cases of pneumonia in both children and adults. Despite advances in diagnostics and therapeutics in recent years, mortality rates have remained unacceptably high.

Causes

The same viral pathogens that cause upper respiratory tract infections in the community during the winter months are detectable in over 70% of CAP cases in children. RSV is the most commonly identified cause of viral pneumonias in children, especially infants, in most communities. Although viruses have long been accepted as the responsible causative

Table 32.3 Most common pathogens of lower respiratory infections by age

Age	Cause
Neonates	Group B <i>Streptococcus</i> <i>Escherichia coli</i> <i>Listeria monocytogenes</i> Cytomegalovirus
Infants	Respiratory viruses (respiratory syncytial virus, parainfluenza virus type 3) <i>Streptococcus pneumoniae</i> <i>Bordetella pertussis</i> <i>Chlamydia trachomatis</i>
5 months–5 years	Respiratory viruses <i>S. pneumoniae</i> <i>Haemophilus influenzae</i> <i>Staphylococcus aureus</i> <i>Mycoplasma pneumoniae</i> <i>Mycobacterium tuberculosis</i>
5 years–18 years	<i>M. pneumoniae</i> <i>S. pneumoniae</i> <i>Chlamydia pneumoniae</i> <i>M. tuberculosis</i>
Young adults (18 years–45 years)	<i>S. pneumoniae</i> <i>S. aureus</i> <i>H. influenzae</i> (type b and nontypeable) <i>C. pneumoniae</i> <i>M. pneumoniae</i> <i>M. tuberculosis</i>
Older adults	<i>S. pneumoniae</i> <i>H. influenzae</i> (type b and nontypeable) <i>M. pneumoniae</i> <i>Legionella</i> spp. <i>M. tuberculosis</i>
Institutionalized adults	Gram-negative bacilli, including Enterobacteriales, <i>Pseudomonas</i> , and <i>Acinetobacter</i> <i>S. aureus</i> <i>S. pneumoniae</i>

agent in many cases of pediatric pneumonia, a new appreciation for their role in adult CAP is evolving in large part because of advanced molecular diagnostic techniques that allow rapid and sensitive virus detection. Viruses were identified in more than 25% of adult pneumonia cases in one multicenter study. Influenza virus can cause primary viral pneumonia, be associated with subsequent bacterial superinfection (i.e., *S. pneumoniae* and *S. aureus*, secondary bacterial pneumonia), or lead to a mixed viral-bacterial pneumonia. Parainfluenza viruses 1 to 3, rhinoviruses, coronaviruses including SARS-CoV-2, hMPV, and adenoviruses are also frequently detected.

The most common bacterial cause of CAP in children and adults is *S. pneumoniae*. Although invasive pneumococcal disease with vaccine serotypes has decreased in children since the introduction of PCV7 and later with PCV13, many studies have demonstrated increased infections with non-vaccine serotypes. In the United States, the proportion of bacterial pneumonias in adults caused by *S. pneumoniae* has decreased significantly since the pre-antibiotic era. In children, other common pathogens include nontypeable *H. influenzae*, *M. catarrhalis*, and *M. pneumoniae*. In neonates, group B *Streptococcus* should be considered a potential pathogen.

In adult patients, *H. influenzae*, *M. catarrhalis*, and atypical pathogens such as *M. pneumoniae*, *C. pneumoniae*, and *Legionella* should also be considered. Patients with chronic lung disease can become infected with *H. influenzae*. *Legionella* more commonly affects older adult patients and those with immunocompromising and underlying comorbidities; the reported incidence has been increasing. Patients with a recent influenza virus infection are at increased risk of developing secondary bacterial pneumonia caused by *S. aureus* or *S. pneumoniae*.

The term *healthcare-associated pneumonia* (HCAP), as defined by the American Thoracic Society and Infectious Diseases Society of America, occurs after 2 or more days in a hospital or health care facility. Patients with HCAP are typically of older age and are infected with MDR organisms such as MRSA and gram-negative bacteria including *P. aeruginosa*. However, studies have not demonstrated the ability of these healthcare-associated risk factors to predict infections with MDR organisms, which appear to be more strongly linked with individual patient comorbidities. It is recommended that individual risk factors for MDR organisms be validated locally and used to guide antimicrobial selection.

Although *S. aureus* accounts for only a small percentage of cases of CAP, these infections deserve special attention because of a high mortality rate. Historically, most patients with *S. aureus* pneumonia were older and had serious underlying disorders such as cardiovascular disease, chronic pulmonary disease, diabetes, or cancer, and strains of MRSA were usually hospital-acquired. However, in recent years, a syndrome of necrotizing pneumonia with pulmonary hemorrhage has been associated with MRSA isolates that produce the Panton-Valentine leukocidin cytotoxin. Interestingly, these strains are often community acquired and can occur in young and otherwise healthy individuals, leading to significant morbidity and mortality.

Endemic mycoses, including histoplasmosis, blastomycosis, and coccidioidomycosis, can also cause acute pneumonia syndromes that may be indistinguishable from a typical bacterial community-acquired pulmonary infection. These infections may go unrecognized in many cases that self-resolve or may be diagnosed only when chronic, progressive, or disseminated disease is present despite antibacterial therapy.

Pathogenesis

It is now realized that the lung is not a sterile site as previously thought; a disruption in the balance of microbes can lead to pneumonia. In cases in which a new pathogen is introduced, bacteria from the oropharynx or GI tract gain access to the lower airways through aspiration. Normally, the defense mechanisms of the lung, including the ciliated epithelial cells and innate immune system phagocytic cells, can clear aspirated bacteria before imbalance in the normal ecosystem develops and infection is established. However, infection of the lower respiratory tract could develop if the bacteria involved are particularly virulent, the inoculum of inhaled microorganisms is high, or the immune system has been compromised. Other mechanisms can include local extension from a contiguous source of infection or hematogenous spread from a remote source of infection. Host variables that can contribute to the likelihood of aspiration and development of pneumonia include an impaired level of consciousness, underlying chronic lung disease, and receipt of immunosuppressive therapy.

Clinical manifestations

The clinical presentation of typical bacterial CAP (e.g., *S. pneumoniae*) in adults differs with the age and immunologic status of the host. Most patients with bacterial pneumonia note a relatively sudden onset of fever associated with chills. Cough producing purulent sputum that may be blood-tinged is typical. Chest examination and x-rays often show a localized area of lung involvement, described as lobar consolidation (Fig. 32.5). This abnormality in the lung tissue may be associated with hypoxia as measured by arterial blood gas monitoring, depending on the extent of lung involvement and existence of underlying lung disease. Routine laboratory studies will usually show an increased WBC count and neutrophilia with an increased percentage of immature forms of granulocytes (termed a *left shift*).

In older or immunocompromised patients, the clinical presentation of acute bacterial pneumonia may be less impressive than in patients who can mount a normal immune response to pulmonary bacterial infection. These immunologically compromised patients may have few respiratory tract

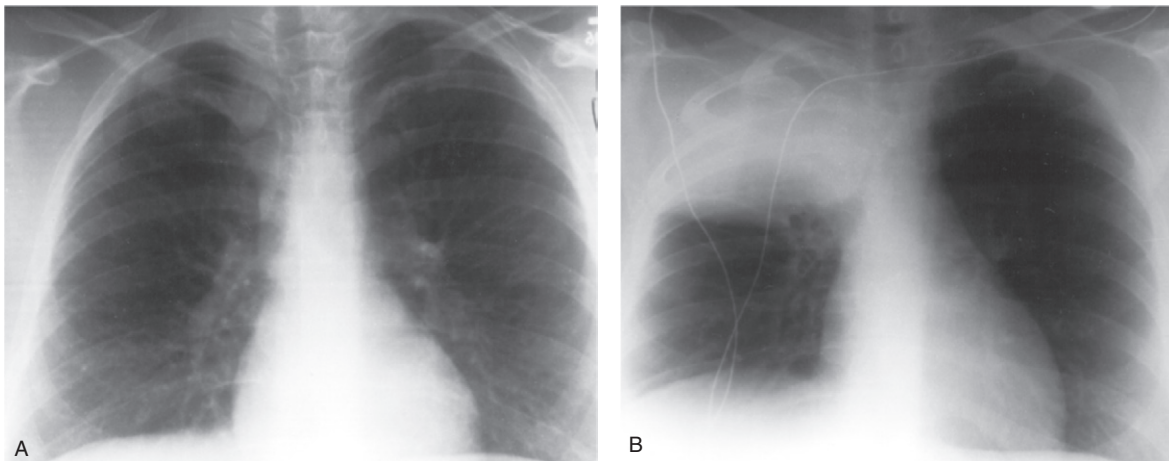


Fig. 32.5 Chest x-rays before (A) and after (B) development of an acute, community-acquired, pneumococcal pneumonia. The patient is facing the reader. B, Consolidation of the upper lobe of the right lung is evidenced by the dense, whitish opacification of this lobe, which contrasts with the normal air (black) density of the remainder of the lung.

complaints and low-grade or no fever. Nonspecific symptoms such as weakness, loss of appetite, and confusion may be the only manifestations of a progressive pneumonia. It is important to maintain a high index of suspicion in these patients and to pursue changes in patterns of behavior routinely with complete evaluations to avoid missing the diagnosis of pneumonia.

Mycoplasma infections may include symptoms of upper respiratory tract infection, rashes, and hemolytic anemia. *Legionella* infections often present with confusion; GI symptoms, including abdominal pain and diarrhea; and a dearth of pulmonary localizing symptoms early on. Hyponatremia is often notable.

Complications

Common complications of CAP include **parapneumonic effusions** (PPEs), which is the accumulation of fluid in the pleural space caused by inflammation from parenchymal lung infection. PPEs occur frequently in the setting of CAP; those that are less severe resolve with antimicrobial therapy. However, if appropriate therapy is delayed, PPEs risk becoming overtly infected, resulting in the formation of empyemas, collections of purulent fluid in the pleural space that require drainage (see the “Empyema” section later in this chapter).

CAP can also result in respiratory failure, including the need for mechanical ventilation; ARDS; and septic shock with multisystem organ failure. The mortality rate for CAP depends on the severity of disease; some studies have found mortality rates in excess of 20% in patients with severe disease. Patients with CAP have higher rates of long-term mortality as well, likely in part as a result of increased cardiovascular complications. A minority of *M. pneumoniae* atypical pneumonia cases result in encephalitis.

Laboratory diagnosis

For patients with mild disease who are managed as outpatients, microbiological diagnostic tests are generally not performed. However, during the COVID-19 pandemic, SARS-CoV-2 testing may be an exception because of the public health implications. Lower respiratory secretions and

blood cultures are recommended for those who are hospitalized and have severe CAP or who have risk factors for infection with MRSA or *P. aeruginosa* (including prior positive cultures and recent hospitalization and IV antimicrobial therapy). For patients who do not require mechanical ventilation, expectorated sputum cultures are easiest to obtain and the least invasive. These sputum cultures are often problematic because sputum expectorated from the lower airways must pass through the upper airway, which is colonized with many bacteria; this can interfere with isolation of the true pathogens and make interpretation of the results difficult. It is often recommended that these cultures be screened to ensure that they have no more than 10 squamous epithelial cells and at least 25 polymorphonuclear cells per low-power field to ensure that the sample is acceptable (Fig. 32.6); samples containing more than 25 squamous epithelial cells per low-power field (Fig. 32.7A) should not be cultured. Cultures obtained from secretions suctioned from a tracheal tube in ventilated patients have many of the same issues because endotracheal tubes and tracheostomies are frequently colonized with bacteria that may be irrelevant for the diagnosis of lower respiratory tract infection.

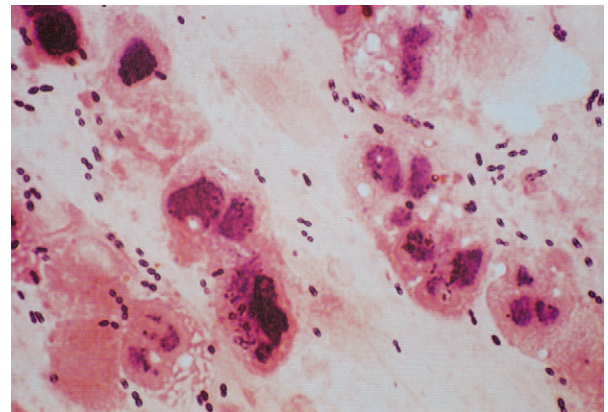


Fig. 32.6 Gram-stained smear of sputum acceptable for culture, with white blood cells and gram-positive diplococci suggestive of *Streptococcus pneumoniae*.

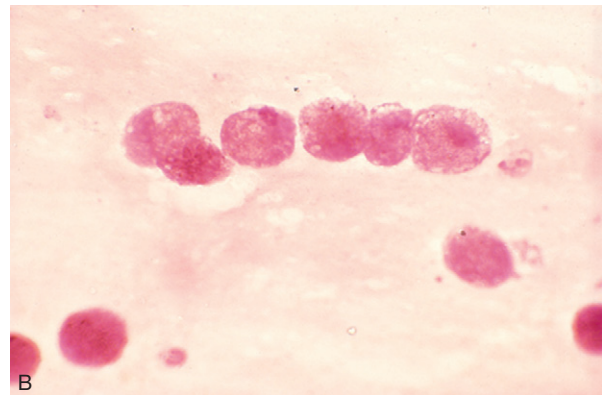
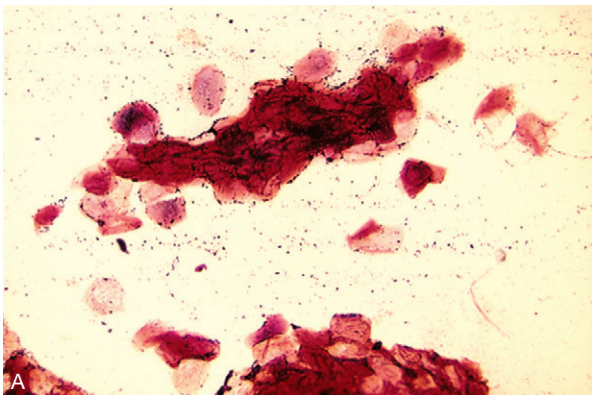


Fig. 32.7 **A**, Expectorated sputum. The Gram-stained smear is visualized with light microscopy under low-power view. There are no white blood cells, but there is a heavy presence of contaminating bacteria and epithelial cells. The sample is saliva, not sputum. There could be several reasons for submission of this sample to the laboratory. The patient could have been poorly directed and simply spit into the collection container, or the patient's cough may not have been productive of sputum. **B**, Aspirated sputum. The Gram-stained smear is visualized with light microscopy under high-power view. There are no white blood cells or organisms. However, it shows specialized cells and mucus (pink-stained background), which are the local materials from the surface of the tracheobronchial tree. This smear confirms that sputum was sampled and that there is no suspicion for infection and no evidence of significant contamination (e.g., large numbers of epithelial cells, commonly found in the mouth). Routine bacterial culture of this specimen might still grow insignificant oral biota or pathogens.

Other more invasive methods of obtaining lower respiratory tract samples include tracheal needle aspiration, which is rarely used because of its invasive nature, risk of complications, and poor acceptability by patients. Bronchoscopic methods are commonly used for patients who require mechanical ventilation as well as in cases of refractory or recurrent pneumonia, when the success of empiric therapy is in doubt. Cultures obtained by these more invasive methods also do not have 100% specificity because upper airway contamination can still occur during specimen collection. Many authorities have recommended a quantitative approach to cultures in which a threshold amount of growth must be present with each of these methods to predict causality of the bacterial growth detected. Quantitation does not apply to certain agents that are considered pathogenic when isolated at any concentration, including *M. tuberculosis*, *Legionella*, certain fungi, and agents of bioterrorism (see later).

In addition to issues of specificity in identifying a true pathogen, sensitivity is also an issue for any of these culture methods. Common pathogens, such as *S. pneumoniae*, are intrinsically difficult to culture, and pretreatment with antimicrobial agents can render cultures falsely negative. *Legionella* cases can also be challenging to diagnose because culture of the organism from sputum or a bronchial aspirate specimen requires special media, and *Legionella* spp. do not stain well with the Gram stain (Fig. 32.8).

Molecular diagnostic tools such as bacterial multiplex PCR assays have been used to identify pathogens in cases in which cultures are otherwise negative and to provide rapid test results to allow better targeting of therapy. Although sensitivity is significantly increased, a PCR has the same problems with specificity, and results must be interpreted in the context of the individual patient to determine whether a true pathogen was detected or if the result represents asymptomatic colonization. In many cases, multiplex molecular assays identify multiple pathogens in the same specimen that may be of uncertain significance.

S. pneumoniae characteristically produces α -hemolytic colonies on sheep blood agar. Biochemical tests, such as bile solubility and optochin sensitivity, can distinguish *S. pneumoniae* from other α -hemolytic streptococci. Cultures from blood and pleural fluid also can indicate the cause of pneumonia. Many adults with pneumococcal pneumonia also have positive blood

cultures (Fig. 32.9); this is less common in children. Adjunctive tests are available for many pathogens. Urinary antigen testing, which is noninvasive and relatively quick to perform, is commercially available for *S. pneumoniae* and *Legionella pneumophila*. Urinary antigen testing for *L. pneumophila* detects only serogroup 1; however, this accounts for the majority of community-acquired cases. Urinary antigen testing is recommended for both pathogens in severe CAP; testing for *Legionella* is also recommended in the setting of an outbreak or recent travel. Both a urinary antigen and a lower respiratory culture or PCR for *Legionella* should be performed when this diagnosis is considered. Testing for *M. pneumoniae* and *C. pneumoniae* can be problematic; options include culture, serologic tests, and PCR assays. A nasal PCR assay for MRSA can facilitate discontinuing or withholding MRSA therapy when negative.

Excellent molecular diagnostics are now available for many commonly encountered respiratory viruses, often as part of a multiplex PCR panel. Testing with a rapid molecular assay to detect influenza is recommended during annual community outbreaks; testing for SARS-CoV-2 is currently also indicated in patients with respiratory illnesses. These tests have excellent sensitivity, but specificity is variable. The presence of a virus does not rule out a concurrent or superimposed bacterial infection, and the result of the viral PCR assay must be interpreted with respect to the clinical situation.

Treatment

An initial decision about management of CAP is whether the patient should be hospitalized or can be treated as an outpatient. Validated clinical prediction rules for prognosis such as the Pneumonia Severity Index (PSI) should be used in addition to an assessment of each patient's resources and clinical situation to determine whether admission is warranted.

Patient comorbidities and the likelihood of drug-resistant pathogens must be considered to determine appropriate empiric antimicrobial therapy. Outpatients without risk factors or comorbidities may be treated with macrolide antibiotics, doxycycline, or amoxicillin monotherapy. Outpatients with certain comorbidities and inpatients with nonsevere CAP are recommended to receive a β -lactam antibiotic plus either a macrolide or doxycycline or a respiratory fluoroquinolone alone. Inpatients with severe CAP can be treated with a β -lactam antibiotic in combination with either a macrolide or a respiratory fluoroquinolone. If locally validated

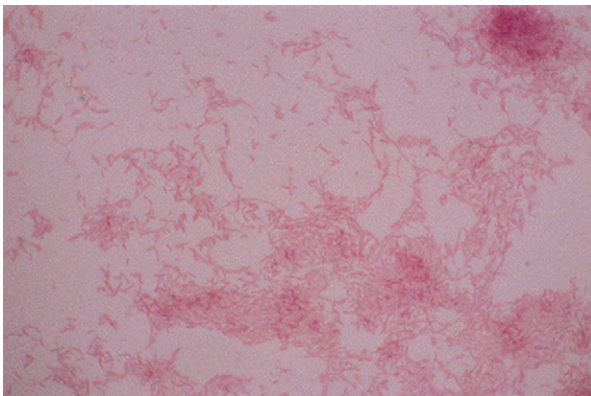


Fig. 32.8 Gram-stained smear of *Legionella* species taken from culture revealing thin, long filamentous, lightly stained, gram-negative bacilli.

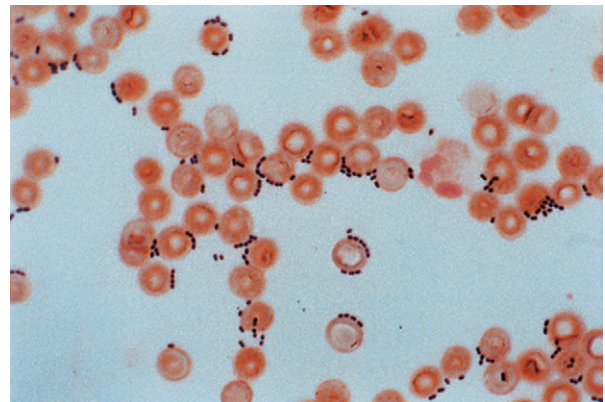


Fig. 32.9 Gram-stained smear of *Streptococcus pneumoniae* isolated from the blood culture of a patient with pneumococcal pneumonia revealing gram-positive cocci and short chains.

risk factors for MRSA or *P. aeruginosa* are present, regimens should be adjusted to empirically cover these pathogens. If cultures obtained do not grow these organisms, antimicrobial therapy should be deescalated.

For young children, most cases of pneumonia are viral in origin, and routine antimicrobial therapy for all cases is not recommended. When antimicrobial agents are indicated, amoxicillin or ampicillin may be used if drug resistance is not a concern, and third-generation cephalosporins are used when significant resistance exists, the child is not fully immunized against Hib and *S. pneumoniae*, or the infection is severe. A macrolide is used alone or in addition to the β -lactam antibiotic when atypical pathogens are suspected in the outpatient and hospital settings, respectively.

A specific pathogen is never identified in the majority of cases, and treatment remains empiric; however, therapy should be pathogen-directed when possible. The duration of therapy depends in part on clinical improvement of the patient but is typically for a minimum of 5 days for uncomplicated adult infections; recommended courses in children may be longer.

Patients diagnosed with influenza A and influenza B pneumonia should be treated with antiviral therapy regardless of the duration of illness before diagnosis. As with many respiratory infections, prevention with vaccination is of critical importance. All individuals older than 6 months of age without contraindications should receive an annual influenza vaccine. Young children and older adults or adults with comorbidities should receive a pneumococcal vaccine. Age-appropriate vaccination for pertussis and Hib is also recommended. Vaccination against SARS-CoV-2 is now available.

Hospital-acquired and ventilator-associated pneumonias

Case study

A 60-year-old male patient with a history of emphysema and chronic bronchitis was admitted to the hospital to have his gallbladder removed. He was given perioperative antimicrobial prophylaxis with cefoxitin. Because of the patient's underlying lung disease, there was difficulty weaning him from the ventilator post-operatively. Seven days after surgery, he developed a high fever, increased secretions from his endotracheal tube, and an infiltrate in the lower lobe of the right lung, as detected on a chest x-ray.

HAP or nosocomial pneumonia is defined as pneumonia that is acquired 48 hours or more after hospital admission and is not associated with mechanical ventilation, whereas **ventilator-associated pneumonia (VAP)** is a pneumonia episode diagnosed at least 48 hours after airway intubation and initiation of mechanical ventilation.

Epidemiology

VAP is estimated to occur in approximately 10% of patients who require mechanical ventilation for longer than 48 hours, and it carries a substantial morbidity and mortality risk. VAP is associated with longer duration of mechanical ventilation, longer hospital stay, and increased health care costs. The all-cause mortality rate has been estimated between 20% and

50%; the attributable mortality in one meta-analysis was 13%. Although less data exist for HAP, it has been associated with significant complications as well.

Causes

The spectrum of pathogens associated with HAP differs from that of CAP. Because microaspiration of upper airway secretions is the most common route of pathogen entry into the lower respiratory tract, the cause of HAP depends largely on the types of organisms colonizing the oropharynx and possibly the GI tract. Patients with HAP are at greater risk for colonization and infection with a wider spectrum of MDR bacterial pathogens, such as MRSA, *P. aeruginosa*, Enterobacterales (e.g., *Enterobacter* spp., *Klebsiella* spp., and *E. coli*, including those producing extended-spectrum β -lactamases [ESBLs] and carbapenemases), and *Acinetobacter baumannii*. The spectrum of MDR pathogens is dynamic and may differ by geographic area, hospital, or specific unit within the hospital. The most consistently identified risk factor for MDR HAP/VAP is recent exposure to antimicrobial agents. The availability of current hospital epidemiologic data on MDR pathogens and their antimicrobial susceptibilities (termed a *cumulative antibiogram*) is essential in choosing appropriate empiric antimicrobial therapy. Mixed infections are common, although anaerobic infections are seen infrequently. Rates of hospital-acquired *L. pneumophila* infection differ considerably among hospitals but are generally lower than for community-acquired infection with this organism. Viruses may be responsible for up to 20% of HAP/VAP cases.

Pathogenesis

As with CAP, the pathogenesis of HAP is related to upper respiratory tract colonization, the number and virulence of organisms entering the lower airway, and the response of the host's defenses to this new introduction or imbalance in the normal lung microbiota. Older age, more severe underlying illness, previous treatment with antimicrobial agents, and manipulations that increase the gastric pH are all associated with increased oropharyngeal colonization with bacteria (Fig. 32.10). Such pathogens may be acquired from the patient's own GI tract, but exposure of patients to the microbial biota encountered during hospitalization provides an exogenous source of microbes that can also be involved in subsequent HAP. Hospitalized patients with altered levels of consciousness aspirate pharyngeal contents into the lung more often than normal volunteers. In addition, intubation of the lower airway greatly increases the risk of developing HAP by bypassing the normal mechanical defenses provided by the glottis and cough reflex. Nasogastric intubation also increases the risk of aspiration resulting from interference with glottic function and increased reflux of gastric secretions into the oropharynx.

Clinical manifestations

Diagnosing HAPs is often challenging. Several noninfectious processes can affect the lungs and cause many of the same clinical signs and symptoms and chest radiographic findings that are seen with pneumonia, making differentiation difficult. A generally agreed-on definition using clinical criteria in many guidelines is a new or persistent infiltrate on chest imaging accompanied by any of the following: fever, elevated or depressed WBC count, increased

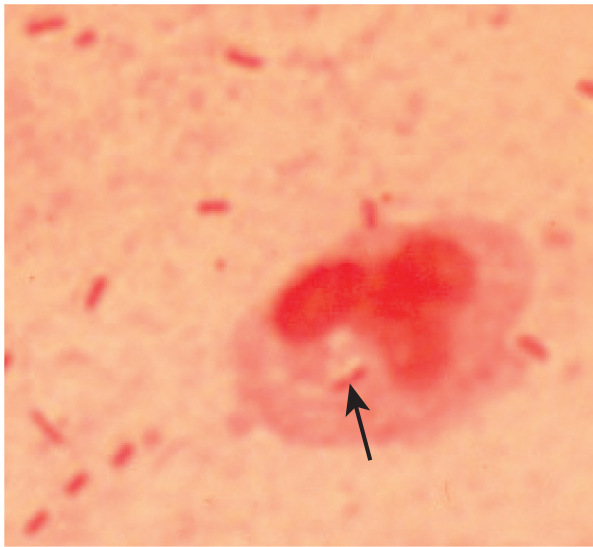


Fig. 32.10 Gram stain of gram-negative bacilli in a tracheal aspirate (the arrow indicates an intracellular gram-negative bacillus). They are common pathogens in hospital-acquired pneumonia. (Courtesy Dr. William M. Janda.)

oxygen requirements, and production of purulent sputum. Unfortunately, all of these findings are relatively nonspecific. Some definitions may also include microbiologic data as a criterion.

Complications

Patients with extensive viral or bacterial pneumonias may have insufficient ventilation to the involved lung to adequately support respiration. In rapidly progressive, multilobar pneumonias, ventilatory support may be needed as a result of overwhelming infection or an injury response of the lung, known as *ARDS*. In these cases, mechanical support of ventilation and other supportive measures are used to provide time for antimicrobial therapy to control the pneumonia and allow recovery of lung function. An example of tissue destruction caused by pneumonia is the development of a lung abscess. In this case, the pulmonary infection results in destruction of the lung parenchyma in the area of a necrotizing bacterial infection. These infections usually can be treated with antimicrobial therapy alone. If the abscess is large and refractory to therapy, surgery may be required. As in CAP, empyemas can also occur.

Extension of the infection beyond the chest usually occurs when bacteria invade the bloodstream (bacteremia). The same type of systemic spread is possible with viral pneumonias (viremia), although the consequences of viremia are usually much less severe than those of bacteremia. Patients with bacteremia as a complication of pneumonia have increased mortality compared with patients with pneumonia without bacteremia. In some cases, this may simply reflect the bacterial burden and the difficulty in controlling the infection. In other cases, such as gram-negative bacillary bacteremia, the presence of bacteria and their constituents (e.g., endotoxin) may be associated with severe shock, further complicating care of the patient and increasing the likelihood of a fatal outcome. In addition to the direct consequences of bacteremia, in some cases, an infection originally established in the lung can spread via the bloodstream to other sites, such as the CNS, a so-called *metastatic infection*.

Laboratory diagnosis

Many of the same principles for microbiologic diagnosis of CAP apply to HAP. Current guidelines for HAP and VAP recommend noninvasive, semi-quantitative sampling (i.e., expectorated sputum or endotracheal aspiration culture) for diagnosis of the potential pathogen rather than invasive sampling with quantitative cultures to avoid unnecessary harm from invasive procedures. More invasive collection techniques such as bronchoscopy may be needed in immunocompromised patients or those who fail to improve with noninvasive testing and therapy. Nasal PCR screening for MRSA can have a high negative predictive value depending on the baseline rate of MRSA and can help deescalate therapy in these situations. Blood cultures are recommended in patients with HAP/VAP, and urine *Legionella* antigen testing and *Legionella* culture should be performed in patients for whom a hospital-acquired *Legionella* infection is a possibility. During community outbreaks, hospital-acquired influenza and SARS-CoV-2 transmission should also be considered.

Case check 32.5

VAP is thought to occur in up to 20% of patients who are mechanically ventilated for more than 48 hours. This patient's underlying chronic lung disease places him at increased risk for this entity as well. The increased endotracheal secretions, fever, and new infiltrate on chest imaging all suggest development of VAP in this patient, who has been mechanically ventilated. Empiric antimicrobial therapy should be started, and a culture of his endotracheal secretions is indicated to hopefully allow future directed tailoring of his treatment regimen. As this is a hospital-acquired infection, the patient is at increased risk for MDR organisms; therefore the facility's antibiogram should be considered when formulating his empiric therapy.

Treatment

Early implementation of appropriate therapy has been shown to decrease mortality in those with HAP/VAP. Broad-spectrum antimicrobial therapy should be started empirically and then modified based on culture data and clinical response. Empiric therapy should include coverage for *S. aureus*, *P. aeruginosa*, and other gram-negative bacilli at a minimum. Coverage for MRSA with vancomycin or linezolid is recommended if the local incidence of MRSA is high (>10% to 20% of *S. aureus* isolates) or unknown, there are risk factors present for MRSA including recent IV antimicrobial use, or there is a high risk of mortality.

Dual empiric antipseudomonal coverage with agents from different classes is recommended when local gram-negative resistance to a single agent is greater than 10% and for patients with structural lung disease, recent IV antimicrobial use, or high risk of death. Once susceptibilities are known, a single antipseudomonal agent can be used. Therapeutic options include extended-spectrum β -lactams, carbapenems, and antipseudomonal fluoroquinolones; aminoglycosides and colistin should be avoided unless no other options exist. For patients in whom *Legionella* infection is suspected, a macrolide antibiotic should be added unless a fluoroquinolone is already being used. A 7-day course of therapy is generally sufficient for HAP and VAP.

There has been significant focus on measures to prevent HAP, especially VAP. VAP intervention bundles, in particular, have been implemented in many hospitals to reduce the rate of VAP. These bundles include measures such as elevating the head of the bed to prevent aspiration, weaning sedation daily to decrease time on the ventilator and prevent complete suppression of the protective cough reflex, and providing oral care to decrease oral colonization with potential pathogens. Although these interventions have been shown to decrease the number of reported VAPs, many have not been shown to improve overall patient outcomes, including mortality.

Empyema

Case study

A 50-year-old male patient was hospitalized with 1 week history of respiratory symptoms and was diagnosed with CAP. Despite treatment with antimicrobials he continued to have low-grade fevers. A chest x-ray showed a left-sided pleural effusion. A CT scan of the chest revealed that the effusion was loculated (not free flowing, but trapped in multiple subcompartments as a result of a fibrous reaction to the infection).

Epidemiology

Empyema is a collection of purulent fluid in the pleural cavity, between the lung and the pleura. Most empyemas occur in association with a concurrent acute CAP, although they can occur with healthcare-associated processes as well. The accumulation of pleural fluid in the setting of pneumonia (PPE) is fairly common; up to 40% of hospitalized pneumonia patients will develop a PPE. However, most such accumulations are sterile, and only a small percentage (5% to 10%) will progress to an empyema.

Risk factors for empyema development include advanced or very young age, male sex, debilitation, comorbid disease(s) including alcohol abuse and IV drug use, and longer duration of symptoms before receiving appropriate therapy. Increases in the incidence of empyema in children have been noted after seasonal and pandemic influenza epidemics. Rates of empyema in the United States seem to be increasing, despite an overall decrease in hospitalizations for pneumonia.

Causes

Because the majority of empyemas are associated with CAP, the pathogens are similar. The most commonly isolated bacteria from empyemas in children and a significant contributor in adults is *S. pneumoniae*. After the introduction of PCV7 and PCV13, rates of invasive pneumococcal disease caused by many of the covered subtypes decreased significantly in both children and adults. *S. pyogenes* and *S. aureus* are also frequently implicated in community infections. Infections associated with aspiration and poor oral hygiene often involve other streptococci and anaerobic bacteria. Empyema among hospitalized patients is increasingly caused by aerobic gram-negative bacilli in addition to *S. aureus*. Empyemas may harbor multiple bacterial species, particularly if they are multiloculated (divided into multiple pleural spaces), caused by aspiration, or from a GI or intraabdominal source.

M. tuberculosis is also an important cause of empyema worldwide.

Pathogenesis

Empyema is usually a consequence of extension of infection directly from the underlying lung to the pleural space. Most empyemas occur in patients with community-acquired infections for whom there has been a delay in treatment. In patients with chronic pneumonias, such as those caused by *M. tuberculosis*, empyema may occur as a result of rupture of an underlying cavity into the pleural space. Empyema may also complicate chest surgery or chest trauma, both of which provide a direct route of infection to the pleural space. Hematogenous seeding of the pleural space can also occur with bacteremia. Less commonly, empyema results from contamination of the pleural space as a result of extension of a pyogenic process from the mediastinum, the paraspinal area, or the abdomen to the pleural space such as after esophageal rupture.

In general, an untreated pneumonia results in increased capillary permeability by active inflammatory cells recruited to the lung because of infection, resulting in fluid accumulation in the pleural space and development of an uncomplicated PPE. If left untreated, fibrin adhesions may develop and bacteria can enter, the end stage of which may be fibrosis and lung entrapment.

Characteristics of the empyema fluid create an environment in which elimination of offending pathogens is compromised. Opsonins and complement activity, which are necessary for proper phagocytosis of bacteria by infiltrating granulocytes, are present in reduced concentrations in empyema fluid. In addition the usefulness of antimicrobials in treating empyema is limited by the minimal blood supply to the area, resulting in reduced drug delivery, and by the low pH of empyema fluid, which can reduce the activity of antimicrobial drugs.

Case check 32.6

The presence of persistent fever despite treatment with appropriate antibacterial therapy suggests a source control problem. Antimicrobial drugs alone are insufficient treatment for a purulent fluid collection, such as an empyema, because blood flow and antimicrobial delivery into such a collection is minimal, resulting in a suboptimal drug concentration at the site of infection; drainage is required. PPEs, such as seen on this patient's chest imaging, should be sampled via thoracentesis, and if the results are consistent with an empyema, drainage with a chest tube or surgical means should be performed. Empyema is more likely with a protracted duration of symptoms before seeking medical care and starting appropriate antimicrobial therapy.

Clinical manifestations

The clinical presentation of a patient with empyema is often indistinguishable from the disease of the underlying lung. Patients can have chest pain on the affected side, fever, chills, and night sweats. Sepsis may develop. If the empyema is large, it might be detected during the physical examination as an area of absent or reduced breath sounds. If empyema is

not suspected based on the physical findings, it can often be detected as an abnormality on the chest x-ray. Pleural ultrasonography, often performed at the bedside, can help determine the feasibility of pleural fluid sampling and facilitate the procedure. A contrasted CT scan of the chest might be necessary to differentiate empyema from a lung abscess or lung consolidation and can help elucidate the etiology of the effusion.

Complications

The main consequence of empyema is persistence of infection. If the empyema is not drained and treated with appropriate antimicrobial agents, the infection causing the empyema might be difficult to eliminate. Persistent, poorly controlled infection can lead to the multiple complications associated with unresolved sepsis. A long-term complication of empyema is encasement of the lung in a thick capsule, which can reduce lung function. This can necessitate removal of the thickened pleural lining (pleural decortication), which may or may not restore the function of the underlying lung, depending on the duration of the dysfunction. Up to 30% of patients with empyema require surgical intervention, and mortality can be up to 15%. Advanced age and major comorbidities are risk factors for death.

Laboratory diagnosis

The distinction between a sterile PPE and empyema depends on the presence of a bacterial pathogen and inflammatory response and is made using pleural fluid cell counts, chemistry tests, and cultures. A thoracentesis (removal of pleural fluid through a needle or tube) is performed to obtain a sampling of the pleural space fluid. Pleural fluid specimens should be aspirated directly from the pleural space (and not from previously placed catheters or tubes) into an evacuated, sterile syringe. Any excess air in the syringe should be expelled promptly to increase the yield of anaerobic bacteria. A portion should also be inoculated into a blood culture bottle to increase the yield of bacteria.

Pleural fluid should be analyzed for cell count with differential, total protein, lactate dehydrogenase (LDH) concentration, and pH in addition to bacterial, fungal, and mycobacterial direct stained smears, and cultures. If malignancy is in the differential diagnosis, a fluid specimen should also be submitted for cytology. If the diagnosis of tuberculous pleural effusion is suspected, measurement of fluid adenosine deaminase may be helpful; a pleural biopsy sample for mycobacterial culture and *M. tuberculosis* PCR assay can increase diagnostic sensitivity. The sensitivity of pleural and blood cultures is variable and often low, especially in patients in whom there has been pretreatment with antimicrobial agents. In these cases, molecular diagnostic techniques may increase the identification of pathogens, such as multiplex PCR testing for common bacteria or 16S RNA PCR to identify pathogens.

Treatment

The mainstay of therapy for empyema is drainage of the infected fluid concurrent with appropriate antimicrobial therapy. Pleural fluid with purulence, a positive Gram-stained smear, or a positive culture requires drainage. A pleural fluid pH less than 7.20, loculated fluid, low fluid glucose, and

elevated fluid LDH level also suggest that the fluid is less likely to resolve with antimicrobial therapy alone and drainage is necessary. Drainage can be performed in many cases with insertion of a chest tube at the bedside or a pigtail catheter with radiologic guidance, potentially with the addition of lytic therapy. For more complicated cases in which these procedures do not drain the fluid, control the infection, and reexpand the lung adequately, video-assisted thoracoscopic surgery or thoracotomy with decortication might be needed. When a specific pathogen is identified from pleural fluid, blood, or respiratory cultures, antimicrobial therapy should be appropriately directed; in the absence of positive cultures, empiric therapy should be continued to cover the most common causes. The duration of therapy depends on the pathogen, adequacy of drainage, and clinical response.

Prevention strategies include appropriate early therapy for pneumonia to decrease the likelihood of empyema development. Vaccination of children and adults against *S. pneumoniae* has been successful in decreasing invasive pneumococcal disease, including empyema, related to the included vaccine serotypes.

Aspiration pneumonia

Case study

A 63-year-old male patient, who was a nursing home resident, was admitted to the hospital for treatment of recurrent urinary tract infections. He had several episodes of vomiting while he was in the nursing home. At the time of admission, he was somnolent, and pulse oximetry revealed hypoxia. A chest x-ray performed by the bedside showed a new infiltrate in the right lung posterior upper lobe. He was transferred to the intensive care unit for management of ARDS.

Epidemiology

Aspiration occurs when gastric or oropharyngeal contents are inhaled into the larynx or lower respiratory tract. Several syndromes can result, including aspiration pneumonitis, a noninfectious chemical injury; and **aspiration pneumonia**, a lower respiratory tract infection caused by inhalation of bacteria colonizing oropharyngeal or, in some cases, gastric secretions. These two syndromes are often difficult to differentiate clinically. In addition, CAP and VAP also likely result from microaspiration of bacteria colonizing the oropharynx.

The aspiration pneumonia discussed in this section will focus on those pneumonias thought to be caused by macroaspiration events. Aspiration pneumonia occurs in children and adults and is more common in hospitalized patients, those with dysphagia, or those who have a reduced level of alertness for a variety of reasons, including alcohol or sedative use. Other risk factors include poor oral hygiene and periodontal disease, esophageal dysmotility disorders, enteral feeding, ineffective cough reflex, and use of gastric acid-blocking medications.

Causes

Traditionally, aspiration pneumonia was thought to be predominantly caused by anaerobic pathogens, but more recent studies have isolated fewer anaerobes. Patients with community-acquired aspiration pneumonia were more likely to have

infections with *S. pneumoniae*, *H. influenzae*, staphylococci, and Enterobacterales, whereas gram-negative pathogens, including *P. aeruginosa*, were isolated more commonly from patients with hospital-acquired aspiration pneumonia. Anaerobic pathogens were isolated infrequently in these newer studies, but they may play a more significant role in patients with prolonged illness and development of complications, including lung abscesses, necrotizing pneumonia, and empyema. The most commonly identified anaerobes include *Bacteroides* spp., *Prevotella*, peptostreptococci, and *Fusobacterium* species.

Pathogenesis

Aspiration pneumonia follows the abnormal entry of upper airway or GI secretions colonized with bacteria into the lower respiratory tract. The likelihood of developing infectious pneumonia after aspiration depends on the frequency of aspiration, quality and quantity of material aspirated, and host defenses. Patients who are immunosuppressed by underlying disease and/or chemotherapy and patients with altered lung defenses (e.g., cigarette smoking, chronic lung disease, advanced age) are at increased risk for development of infection after aspiration of a bacterial inoculum. In addition, inhaled particulate matter can obstruct the airway, causing reduced clearance of bacteria and secretions, thereby predisposing to infection.

Chemical aspiration pneumonitis is thought to occur when inhaled gastric acid causes injury resulting in a severe inflammatory response and increased permeability of the tissue, potentially leading to edema, decreased lung compliance, and hypoxemia. Most of these cases are thought to be sterile, but later secondary bacterial superinfection can occur.

Case check 32.7

Microaspiration events occur even in healthy individuals but are much more common in patients with altered levels of consciousness and difficulty swallowing. Macroaspiration can occur in the setting of vomiting such as in this case. The acidic gastric contents can cause a chemical pneumonitis that can manifest itself with a relatively quick onset of hypoxemia and respiratory failure with fever and a new infiltrate on chest x-ray. This can be very difficult to differentiate from aspiration pneumonia in which there is an infection of the lung tissue after an aspiration event. This patient's residence in a nursing home places him at increased risk of colonization with MDR organisms in his oropharynx that can then be aspirated and cause infection.

Clinical manifestations

Symptoms of aspiration may be evident as coughing while the patient is eating food or drinking liquids or after an episode of emesis, although in many patients, these aspiration events are inapparent or "silent;" in other cases, the aspiration event is unwitnessed. It is often impossible to distinguish aspiration pneumonitis and aspiration pneumonia on clinical presentation alone because there is significant variability and overlap in clinical manifestations. Aspiration pneumonitis classically presents with a witnessed aspiration event and sudden increased coughing, wheezing, hypoxia, and possible progression to respiratory failure. These patients may have fever and significant infiltrates on chest radiography, despite having no active infectious process.

Aspiration pneumonia may present in a similar fashion with acute onset of fever, cough, and shortness of breath, or it may be more subtle. On chest x-ray, disease resulting from aspiration is more likely to involve parts of the lung that are dependent at the time of aspiration, usually the superior segments of the lower lobes and posterior segments of the upper lobes when the patient is supine. The lower lobes are more likely involved when the patient is upright, and right-sided lung involvement is twice as common because the right mainstem bronchus has a more direct course into the lower lobe of the lung.

Complications

Patients diagnosed with aspiration pneumonia carry a higher risk of morbidity and mortality compared with those diagnosed with non-aspiration CAP, even in the absence of other complications. If treatment fails, aspiration pneumonia can progress to a necrotizing pneumonia and lung abscess. Patients with lung abscess usually have a longer history of illness (weeks) and a more subtle onset of symptoms than patients with common acute bacterial pneumonia. Patients with lung abscess are also more likely to have putrid sputum and to complain of halitosis resulting from involvement of anaerobic bacteria in the lung infection. As with other types of pneumonia, empyema can also develop. Acute aspiration pneumonitis can rapidly lead to increasing hypoxia and ARDS.

Diagnosis

Obtaining specimens for the diagnosis of aspiration pneumonia is similar to methods described for CAP and HAP. The difficulty lies in distinguishing aspiration pneumonitis, a noninfectious process that does not benefit from antimicrobial administration, from aspiration pneumonia, which does warrant therapy. Several biomarkers have been studied in respiratory samples and serum to try to distinguish these two entities, but currently no reliable method exists.

Treatment

Treatment for acute aspiration involves airway clearance, oxygen and respiratory support, and treatment of bronchospasm and airway edema as needed. Empiric therapy for community acquired aspiration pneumonia should cover similar pathogens as in other CAPs; empiric therapy in a hospitalized patient should include broad-spectrum antimicrobials that provide coverage for aerobic gram-negative organisms. Anaerobic coverage should be included if risk factors are present (e.g., significant periodontal disease, necrotizing infection, lung abscess). Therapy should later be tailored to cover specific pathogens identified during culture. The duration of therapy is generally 5 to 7 days unless the infection is complicated (e.g. empyema, abscess). Evaluation by a speech therapist may be indicated to assess any impairment of swallowing mechanisms and silent aspiration. Preventive measures include good oral hygiene and positional feeding, although results of studies have been mixed.

Tuberculosis and other chronic infections

Bacterial pneumonias usually resolve completely over a period of weeks. On occasion, however, resolution of pneumonia is delayed, with radiographic lung abnormalities that persist beyond the alleviation of clinical symptoms. Some bacteria that typically cause acute pneumonias induce

necrotizing processes in the lung that can be slow to resolve, such as anaerobic lung infections, gram-negative bacillary pneumonias, and pneumonias caused by *S. aureus*. Some pneumonias are inherently slow in progression and chronic in nature; the most common of these are mycobacterial and fungal infections of the lung.

Case study

A 60-year-old male patient entered the emergency department complaining of cough and fever lasting several weeks. He reported night sweats and weight loss over the past few months. He admitted to heavily drinking alcohol on weekends and to staying in a homeless shelter most of the previous winter. The patient had a fever and was very thin. He coughed frequently during the examination. His chest x-ray revealed a right lung upper lobe infiltrate, with a small area of cavitation.

Epidemiology and causes

Mycobacteria are the most common pathogens causing chronic lower respiratory tract infections in immunocompetent hosts. Tuberculosis (TB), a disease caused by infection with *M. tuberculosis*, was responsible for approximately 10 million new cases in 2020 and 1.5 million deaths annually. In the United States, 7,860 cases of TB were reported in 2021, but up to 13 million people in the United States have latent TB infection. Most TB cases in the United States are diagnosed in foreign-born persons. The remainder of cases tend to occur among socially disadvantaged, older adult or very young, and immunocompromised patients. Substance abuse is an important behavioral risk factor in the United States. Infection with HIV is the most significant risk factor for TB infection reactivation or progression of primary TB infection globally, including in children. Infections with nontuberculous mycobacteria (NTM; e.g., *Mycobacterium avium* complex, *Mycobacterium kansasii*, *Mycobacterium abscessus*) can present with cavitary lung disease that is indistinguishable from TB but usually presents as nodular or patchy lung infiltrates superimposed on underlying lung disease (e.g., bronchiectasis, emphysema, pulmonary fibrosis, cystic fibrosis).

Opportunistic fungal pathogens, such as *Aspergillus* and *Cryptococcus*, can cause acute and chronic lower respiratory tract infections in immunosuppressed patients, and in the case of *Aspergillus*, in those with chronic underlying lung disease, but they rarely do so in immunocompetent hosts. In contrast, the fungal pathogens *Histoplasma capsulatum*, *Blastomyces dermatitidis*, and *Coccidioides immitis* can cause acute and chronic lower respiratory tract infections in immunocompetent hosts; only the chronic forms will be considered in this section. *Histoplasma* is endemic to the Mississippi and Ohio River valleys and a few Mideastern states, whereas *Blastomyces* is usually seen in regions adjacent to the Great Lakes and along the Ohio, Mississippi, and St. Lawrence riverways. *Coccidioidomycosis* exposure occurs in the southwestern United States.

Pathogenesis

Most of these organisms are acquired via inhalation. For *M. tuberculosis*, the organism becomes airborne when an infected person coughs, sneezes, or speaks. Fungal and NTM organisms

are acquired from environmental reservoirs. Chronic infections caused by mycobacterial or fungal pathogens typically elicit a granulomatous response in the lung that is distinct from the acute inflammatory response seen with acute bacterial pneumonias. Both humoral (specific antibody) and cellular (delayed-type hypersensitivity) responses are elicited by these pathogens. The cellular immune response to chronic lung infections is involved in both protective and tissue-destructive aspects of these illnesses.

Clinical manifestations

Chronic histoplasmosis and blastomycosis infections are often clinically indistinguishable from pulmonary TB, presenting with protracted productive cough, fevers, night sweats, weight loss, and malaise. Blastomycosis also can present characteristically as a pulmonary mass lesion, mimicking lung cancer with a nonproductive cough and few additional symptoms. Histoplasmosis can cause hilar and mediastinal lymphadenopathy, in some cases with compressive symptoms of other mediastinal structures secondary to lymph node enlargement. TB in children may be particularly difficult to recognize as the clinical presentation may be nonspecific in comparison with acute bacterial pneumonia; involvement of the peripheral lymph nodes is not uncommon and, when present, may provide a clue.

Case check 32.8

The symptoms seen in the setting of acute pneumonia (e.g., fever, chills, productive cough) can also be observed in patients with chronic pneumonias, but these and other symptoms may be less intense and less dramatic in onset. This can result in a greater delay between symptom onset and diagnosis. Because of the prolonged period of illness associated with chronic pneumonias, the patient can also exhibit signs that are not seen with acute infections, such as weight loss and slowly increasing debility. In patients with cavitary lung lesions, hemoptysis can occur. This manifestation was illustrated in the case presented at the beginning of this section. Upper lobe cavitary lung disease is suggestive of TB or other mycobacterial disease. However, because many infections that cause chronic pneumonia present with similar radiographic abnormalities, further studies must be performed for a specific diagnosis.

Mycobacterial and fungal infections are more likely to be severe and disseminated in immunocompromised patients, compared with immunocompetent hosts, potentially causing life-threatening complications. For example, *Aspergillus* spp. can cause overwhelming pneumonia in neutropenic patients, and cryptococcal pneumonia can cause fatal dissemination to the CNS in patients who are immunosuppressed after receiving chemotherapy or as a result of advanced HIV infection. Physical examination of patients with chronic pneumonia may reveal few abnormalities other than those associated with general debilitation. If dissemination is present, many of the discussed fungal infections may also involve the skin, which can provide clues to the cause and another area for tissue sampling and diagnosis. In addition to the history, chest radiography and CT of the chest are the most important initial diagnostic studies in establishing a presumptive diagnosis of chronic pneumonia.

Complications

The complications of chronic infectious pneumonias depend on the extent of the local and systemic spread of the infection, duration of the illness, and immunologic status of the host. Patients who develop extensive lung involvement with a chronic infection as a result of late, inappropriate, or failed medical intervention can eventually experience progressive debility and respiratory insufficiency. Infection dissemination beyond the lung in immunocompromised patients can result in infection of other vital organs and tissues, such as the CNS with cryptococcus, the endemic mycoses, and *M. tuberculosis* and the bone marrow, spleen, liver, and GI tract with histoplasmosis. Blastomycosis can spread to the skin, bones, and genitourinary tract, even in immunocompetent individuals, and causes ARDS in a subset of patients. Extrapulmonary spread generally increases the morbidity and mortality of these infections.

Laboratory diagnosis

Mycobacterial culture remains the gold standard for diagnosis of TB. Both automated systems with liquid media as well as solid media cultures are recommended to be performed. Liquid media cultures often yield growth faster, but they may be subject to contamination, and some isolates only grow on solid media; using both methods together provides the highest sensitivity. Once growth is seen, molecular diagnostic probes can be used for rapid mycobacterial species identification in many instances; matrix-assisted laser desorption/ionization–time-of-flight (MALDI-TOF) mass spectrometry can also be used. Phenotypic drug sensitivity testing for first-line drugs should be performed on all isolates.

Smears of concentrated sputum samples prepared for mycobacterial culture should also be stained with acid-fast stains, such as Kinyoun or Ziehl-Neelsen stain or with a rapid fluorochrome stain to detect acid-fast bacilli (AFB) (Fig. 32.11). It is strongly recommended to test three separate 5- to 10-mL sputum samples to increase the sensitivity of detection; if sputum cannot be obtained including with induction techniques, flexible bronchoscopic sampling is recommended.

To complement the AFB stain of sputum specimens for rapid diagnosis, NAATs are approved for *M. tuberculosis* lung infections. NAATs use PCR assays and other methods to amplify and detect *M. tuberculosis* DNA and RNA in sputum, thus providing highly sensitive and specific methods to detect *M. tuberculosis* days to weeks before culture positivity.

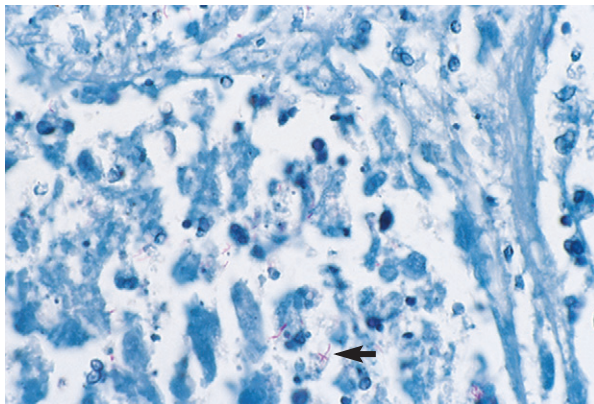


Fig. 32.11 Acid-fast stain of sputum containing mycobacteria (red-stained organisms; arrow).

The sensitivity of these tests is greater for AFB smear-positive sputum than for smear-negative sputum. NAATs are recommended for all sputum specimens that exhibit AFB on direct sputum smears and for AFB smear-negative sputum when the clinical suspicion of TB is high. Molecular assays have been developed that, in addition to detecting the presence of *M. tuberculosis* in sputum specimens, can rapidly detect the presence of rifampin resistance (and in some cases resistance to other drugs), which can be critical to treating drug-resistant TB cases appropriately and expediently; genetic drug susceptibility testing is recommended in those who have risk factors for rifampin resistance.

Unfortunately, because of the paucibacillary nature of TB in children, each of the aforementioned diagnostics has a decreased sensitivity in this population, so TB is often a clinical diagnosis. Obtaining good-quality specimens in young children can also be a challenge. First-morning gastric aspirates can be used to collect swallowed sputum for TB testing when other forms of respiratory specimen collection is limiting. Extrapulmonary TB can be diagnosed using similar techniques as for pulmonary specimens, although the yield is often less. There is less experience with the use of NAATs for TB diagnosis at extrapulmonary infection sites; however, current guidelines recommend its use. Cell counts, chemistries, and adenosine deaminase can be determined on pleural, pericardial, peritoneal, and cerebrospinal (CSF) fluids. Histologic examination may also be helpful for extrapulmonary tissue samples to identify necrotizing granulomas and the presence of AFB organisms on special stains.

Detection of latent TB infection (LTBI), asymptomatic infection without active disease, identifies patients who can be evaluated for treatment to prevent progression to active disease. This may avert both morbidity and mortality in the index patient as well as eliminate the opportunity for transmission to others if effective. Persons who are at low risk for *M. tuberculosis* infection and low risk for development of active disease if infected should not be tested for LTBI. Two tests currently exist to diagnose prior TB exposure: a tuberculin skin test (TST) using purified protein derivative (PPD) and interferon gamma release assays (IGRAs). A positive test result must be followed by an assessment for signs or symptoms of active TB, including imaging of the chest. Patients with clinical symptoms or suspicious findings by chest radiography require assessment with sputum samples to ensure that no active disease is present. Although both PPD and IGRAs are approved for use in detecting LTBI, neither is sufficiently sensitive to rule out the diagnosis of active TB based on a negative test as false-negative results are not uncommon in active disease.

Because NTM can colonize the respiratory tract in the absence of invasive lung disease, more than one positive sputum specimen for the same species in the context of a progressive clinical and radiographic pulmonary process in the absence of another pathogen is generally needed to establish an association between the NTM infection and the illness. Therefore isolation of an organism (e.g., *M. avium* complex) from a single sputum specimen is generally insufficient to establish a causative diagnosis or warrant institution of therapy.

For fungal infections, isolation in culture is the gold standard. Brain heart infusion agar with 10% sheep blood is used for isolation of the more fastidious dimorphic fungi (e.g., *B. dermatidis*, *C. immitis*, and *H. capsulatum*). Sabouraud's dextrose agar is often used for less fastidious fungi like

Cryptococcus spp. Chronic pulmonary histoplasmosis has a higher yield from respiratory culture than acute disease. Fungal cultures of the blood and bone marrow may be relatively high yield in disseminated histoplasmosis. Respiratory cultures (sputum or bronchoscopy) are often able to isolate *Blastomyces*. *Coccidioides* species may be difficult to culture as most patients have a nonproductive cough. Tissue specimens obtained by transbronchial biopsy at bronchoscopy or lung biopsy are ideal to demonstrate the invasiveness of *Aspergillus* because a positive respiratory culture alone may simply represent airway colonization; a presumptive diagnosis is often made clinically. Direct microscopic examination of respiratory secretions should also include preparations to detect fungal agents; potassium hydroxide and calcofluor white are commonly used to detect yeast cells and hyphal elements.

In tissue specimens, additional stains, such as Gomori methenamine silver (GMS) (Fig. 32.12) or periodic acid–Schiff (PAS), may provide a presumptive diagnosis. *B. dermatitidis* is identifiable as a thick-walled large yeast with characteristic, single, broad-based budding. *H. capsulatum* is a smaller yeast with narrow-based budding. Depending on the severity of the clinical illness, empiric therapy is often started based on a presumptive diagnosis from the clinical presentation and results of histopathology studies and special stains while awaiting culture confirmation from the microbiology laboratory because, like TB, many of these cultures grow very slowly, and isolation may take weeks.

Fungal antigen and antibody testing can be a useful adjunct to microscopic studies for the diagnosis of fungal infections pending culture confirmation. Urinary antigen assays for *H. capsulatum* and *B. dermatitidis* have sensitivities greater than 90% in certain populations and presentations of disease; antigen testing can also be used in cerebrospinal and bronchoalveolar lavage fluids. Serum antigen testing for *H. capsulatum* may help increase sensitivity when the clinical suspicion is high. In both of these endemic mycoses, serial monitoring of urine antigen levels can be used to monitor response to therapy and

assess for relapse. A serum antigen assay can detect *C. neoformans*. Serum and bronchoalveolar lavage galactomannan assays may aid in the noninvasive diagnosis of *Aspergillus* infections. Cross-reactivity with other fungal pathogens exists with many of the antigen tests. Serologic tests are used to aid in the diagnosis of coccidioidomycosis and some presentations of histoplasmosis. NAATs are not standardized or widely available but hold promise for the future.

Culture studies in patients with chronic pneumonias require close communication between the clinician and clinical microbiologist. It is necessary to use special techniques to process respiratory secretions and lung tissue specimens for the identification of mycobacterial and fungal pathogens. Furthermore, in some cases (e.g., TB, coccidioidomycosis), isolation of the pathogen creates a potential biohazard in the diagnostic laboratory if the appropriate precautions are not employed. Because of the public health implications of the potential transmissibility of laryngeal or pulmonary TB, any positive respiratory mycobacterial smear or culture should be reported to the primary care provider and local public health departments, and proper airborne isolation precautions should be instituted in health care facilities until the organism is speciated to discriminate between TB and NTM infection, the latter of which is not communicable from person to person and does not require isolation.

Treatment

The presence of *M. tuberculosis* in expectorated sputum is diagnostic and must be treated. Therapies vary in duration, drugs prescribed, and dose and frequency. Regimens for active disease and LTBI also vary. Patients with pansusceptible TB receive a four-drug regimen of rifampin, isoniazid (also known as *isonicotinic acid hydrazide* [INH]), pyrazinamide, and ethambutol (RIPE TB regimen) for an initial 2-month induction period. Once the isolate has been confirmed to be susceptible to INH and rifampin, pyrazinamide and ethambutol therapy can be discontinued. The initial 2-month intensive phase is

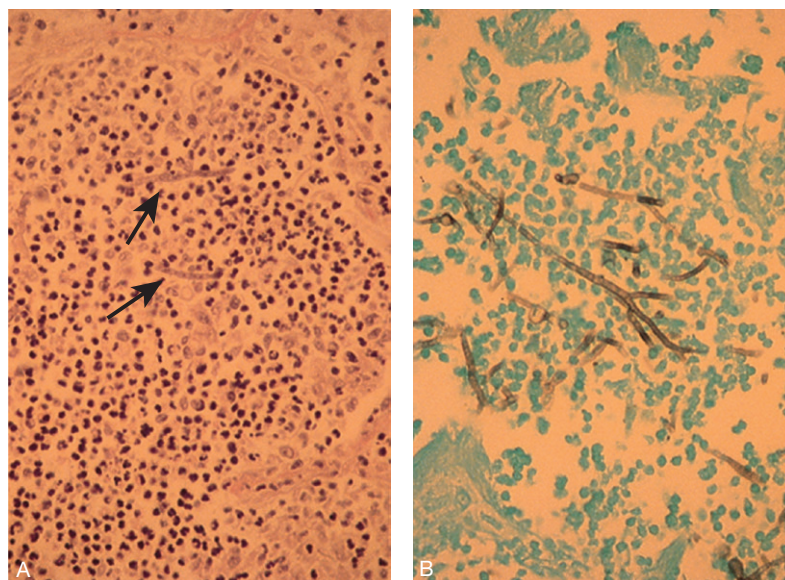


Fig. 32.12 Lung exudate from a patient with a hematologic disorder showing alveoli containing branching fungal elements. **A**, Hematoxylin and eosin stain. Arrows indicate fungal elements. **B**, Gomori methenamine silver stain. (Courtesy Dr. Shirlyn B. McKenzie.)

followed by therapy with INH and rifampin for an additional 4-month or 7-month continuation phase. Patients with cavitary disease and persistent *M. tuberculosis* isolation in culture require longer courses because of a higher risk of relapse.

Ideally, therapy is delivered using what is termed *directly observed therapy* (DOT) because of problems associated with failure of patients to adhere to the prolonged treatment. Poor patient compliance can result in treatment failure and emergence of TB drug resistance because of inadequate therapy and resulting bacterial selection. In addition, DOT facilitates close monitoring for drug side effects including hepatotoxicity. Treatment duration must be prolonged (15–24 months) for MDR TB, which is defined as resistance of the *M. tuberculosis* isolate to at least INH and rifampin. In these cases, therapy should always be based on antimicrobial susceptibility testing results and initiated after consultation with a specialist in TB therapy. Extrapulmonary TB that involves the CNS and bone also warrants a longer course of therapy. Treatment of LTBI is critical to preventing progression to active disease, especially in high-risk groups, such as those with HIV infection. This generally involves the use of rifampin alone for 4 months, rifapentine with INH for 3 months, or 3 months of rifampin with INH. In patients with active TB, treatment of comorbid HIV infection is paramount.

Therapy for NTM varies based on the specific mycobacteria isolated as well as the clinical condition of the patient, and single positive NTM cultures are usually an inadequate basis for initiation of therapy; repeated culture confirmation is useful. Multiple agent regimens for a prolonged period are often necessary. Cure of the infection is not always a realistic goal, and treatment in many cases is directed at control of symptoms. In a subset of cases, the toxicity of therapy may outweigh the expected benefits, and close observation may be the most appropriate approach.

Chronic pneumonia and disseminated disease caused by fungal infection also require prolonged therapy. In cases of severe illness, therapy can be initiated with an IV lipid amphotericin B preparation and then followed with an oral azole agent for prolonged treatment (minimum of 12 months); oral therapy can be used as initial treatment for patients with mild-to-moderate illness. The oral antifungal agents of choice depend on the fungal pathogen being treated. For example, itraconazole is often useful for the treatment of infections with *H. capsulatum* and *B. dermatitidis*, but voriconazole is the oral agent of choice for invasive *Aspergillus* infection. Fluconazole has excellent activity against *Coccidioides*. For many of the azoles, therapeutic drug monitoring is critical to ensure absorption and appropriate drug levels for treatment.

Respiratory tract infections in the immunocompromised host

Opportunistic pathogens generally do not cause significant disease in hosts with normal immune system function but rather require an impairment of host defense mechanisms to cause disease; patients who are susceptible to such opportunistic infections are referred to as *immunocompromised hosts*. The expanded use of chemotherapeutic regimens for malignancy, increasing use of solid organ and hematopoietic cell

transplantation, prolonged survival of patients with congenital immune deficiencies and autoimmune disorders, and the HIV/AIDS epidemic have all contributed to increasing numbers of immunocompromised hosts. Pulmonary infection is the most common form of documented infection in these individuals. Because early diagnosis and prompt, directed therapy are the cornerstones of successful treatment of infections in immunocompromised patients, the clinical microbiology laboratory plays an important role in their management.

Patients with human immunodeficiency virus

Case study

An 18-year-old male patient with no significant medical history presented to the emergency department with a history of cough and shortness of breath with exertion, along with subjective fevers, chills, and fatigue. He was noted to be hypoxic. A chest x-ray showed bilateral infiltrates in a diffuse butterfly pattern involving both central lung fields. The patient reported a history of IV drug use, with frequent sharing of needles.

The occurrence of infections in patients with HIV is a combined function of the extent of underlying host immunodeficiency and pathogen virulence. The CD4 cell count remains the best surrogate marker for assessing the status of the host immune system and associated risk of infectious complications. With higher CD4 cell counts, it is more likely that infections will be caused by more virulent organisms, such as *S. pneumoniae* or *M. tuberculosis*, that are also capable of causing disease in immunocompetent hosts. As the CD4 cell count decreases, the risk increases for infection with environmentally ubiquitous opportunistic pathogens that rarely cause disease in normal hosts, including *Pneumocystis jiroveci*, and dissemination of infections that would otherwise be contained locally in a healthy individual (Table 32.4).

Epidemiology

With the availability of highly active antiretroviral therapy (HAART) as well as preventive antimicrobial therapy, the incidence of most infections in patients with HIV has significantly decreased in high-income countries. Nevertheless, respiratory infections remain a common occurrence, especially in patients with very low CD4 cell counts who are not receiving effective antiretroviral therapy (ART). *P. jiroveci*, causing *Pneumocystis pneumonia* (PCP), remains the most common AIDS-associated opportunistic infection, with most cases occurring when the CD4 cell count is below 200/mm³. Initial *Pneumocystis* infection occurs in early childhood, and disease in HIV-infected individuals constitutes both reactivation of latent infection as well as new infections from airborne spread. Other fungal pulmonary infections in this population include infections with the endemic fungi *H. capsulatum* and *C. immitis/Coccidioides posadasii*, both of which cause disseminated disease with increased incidence in patients with advanced HIV infection. *C. neoformans* is an encapsulated fungus that is widespread in the environment and usually causes meningitis, meningoencephalitis, or disseminated disease in AIDS patients with CD4 cell counts less than 100/mm³, but

Table 32.4 Respiratory pathogens associated with absolute CD4 cell count in patients with HIV infection

Pathogen	CD4 cell count (/mm ³)
Bacteria	
<i>Mycobacterium tuberculosis</i>	Any
<i>Mycobacterium avium</i> complex	<50
<i>Streptococcus pneumoniae</i>	Any
<i>Haemophilus influenzae</i>	Any
Protozoa	
<i>Toxoplasma gondii</i>	<100
Fungi	
<i>Pneumocystis jiroveci</i>	<200
<i>Coccidioides immitis</i>	<250
<i>Histoplasma capsulatum</i>	<150
<i>Cryptococcus neoformans/gattii</i>	<100
Viruses	
Influenza virus	Any
Cytomegalovirus	<50
<i>HIV</i> , Human immunodeficiency virus.	

localized pulmonary disease also occurs. *Cryptococcus gattii* is less common and, in the United States, has been identified primarily on the West Coast. Invasive *Aspergillus* fungal infections are rare and often associated with other risk factors, including neutropenia and corticosteroid use.

Patients with HIV infection with preserved CD4 cell counts still have an increased risk of several respiratory infections compared with individuals not infected with HIV. Bacterial pneumonia is common, occurring with increasing frequency at all CD4 cell counts. The same pathogens causing the most diseases in immunocompetent hosts (*S. pneumoniae* and *H. influenzae*) are responsible in HIV-infected patients as well. *P. aeruginosa* and *S. aureus*, however, are seen with increasing frequency in this group in community-acquired infections. Influenza is a common cause of upper respiratory tract infections and bronchitis; cases of influenza pneumonia may be complicated by bacterial superinfection with *S. pneumoniae* and *S. aureus*.

TB is one of the most common HIV-related opportunistic infections worldwide and is a major cause of death, especially among those with advanced HIV disease. TB can occur at any CD4 cell count level, but those with advanced HIV infection are more likely to develop TB disease and disseminated infection. Because patients with HIV infection have a significantly increased risk of progression of recent TB infection to active disease and of reactivation of LTBI, the diagnosis of TB remains an important consideration, even in areas of the world with a low TB prevalence.

Clinical manifestations

PCP tends to have a subacute onset of progressive symptoms, including dyspnea, dry cough, and fever. Hypoxemia is characteristic of PCP and helps categorize the severity of the disease. In general, HIV-infected individuals with CD4 cell counts of at least 300/mm³ are more likely to have histoplasmosis limited to the respiratory tract, whereas disseminated histoplasmosis more commonly occurs in HIV-infected

Case check 32.9

In the Case Study at the beginning of this section, the patient has a history of IV drug use, which is a risk factor for HIV infection. As noted, the incidence of most infections in HIV-infected patients has significantly declined, although it remains a common occurrence in patients with very low CD4 cell counts. PCP remains the most common AIDS-associated opportunistic infection. Patients with PCP classically present with a subacute onset of progressive dyspnea, fever, and nonproductive cough. Hypoxemia, by pulse oximetry or arterial blood gas measurement, is a classic finding. Chest imaging usually reveals diffuse bilateral lung changes, although the radiographic presentation can vary widely. Samples for laboratory testing should be taken from such patients to identify and confirm the diagnosis of HIV infection and the cause of the respiratory disease.

patients with CD4 cell counts less than 150/mm³, and it can involve the lungs, skin, bone marrow and reticuloendothelial system, GI tract, and CNS. Symptoms may include fever, weight loss, cough, dyspnea, diarrhea, abdominal pain, and headache, depending on the areas affected. If the infection involves the bone marrow, cytopenias may be evident on laboratory blood tests. Coccidioidomycosis can have diverse presentations, ranging from focal or diffuse respiratory disease to meningitis and other systemic involvement. As with many other infections, disseminated disease is more common with advanced immunosuppression (CD4 cell count <250/mm³). Cryptococcal pulmonary infection manifests itself with cough and dyspnea, which is indistinguishable from other pulmonary infections; it can also manifest as ARDS in some cases.

As in immunocompetent individuals, bacterial pneumonias present with an acute onset of respiratory symptoms, such as fever, chest pain, and productive cough. The clinical manifestations of TB depend on the extent of disease. Disease limited to the lung may result in a chronic cough with fevers and night sweats similar to those in immunocompetent hosts, whereas disseminated disease can result in progression to sepsis.

Laboratory diagnosis

Because of the large differential diagnosis that often exists for HIV-infected patients with respiratory infections and the importance of timely directed therapy, establishing the diagnosis is crucial. For patients who are actively coughing or for whom sputum induction methods are fruitful, sputum samples can be used for many of these tests. If a specific diagnosis is not established, bronchoalveolar lavage can be used to identify HIV-related pathogens in samples from the lower respiratory tract.

P. jiroveci identification in respiratory secretions is usually done by direct immunofluorescent antibody (DFA) testing, staining with one of many methods to detect the cysts (Fig. 32.13), or PCR assay. Serum β -D-glucan testing, while nonspecific, can be useful as an adjunct or screen. Elevated serum LDH levels are also commonly seen but are also nonspecific.

Histoplasma antigen can be detected in the urine and serum of most patients with disseminated infection and diffuse acute pulmonary histoplasmosis. In addition, the *Histoplasma* antigen can be used to monitor response to treatment. *H. capsulatum* can also often be cultured from respiratory secretions, blood, and bone marrow, although cultures need to be held for at least 4 weeks. Pathology specimens should be stained and examined for characteristic yeast forms.

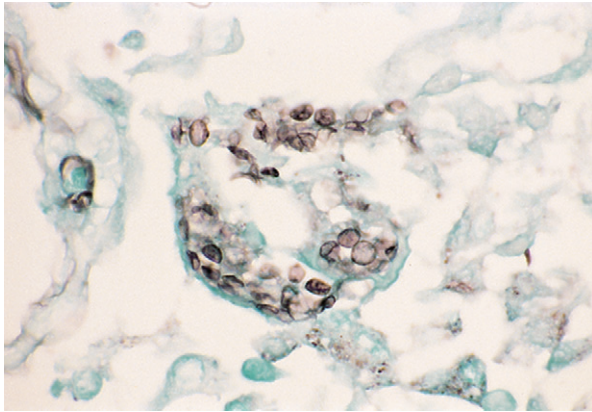


Fig. 32.13 Gomori methenamine silver stain of bronchoalveolar lavage with *Pneumocystis jirovecii* (black-stained cysts).

Coccidioidomycosis is often tested for using serum and CSF, although these tests less often give positive results in immunocompromised individuals. When positive, complement fixation serology can be used to follow response to therapy. Positive culture of clinical specimens, including respiratory secretions, or demonstration of characteristic spherules in tissue specimens can make a definitive diagnosis of coccidioidomycosis. This pathogen is of particular importance for the laboratory because if it is not handled with appropriate precautions, it can become aerosolized and inhaled by laboratory staff, resulting in acute infection. It is important that primary health care providers notify the laboratory when this agent is suspected. Urine and serum antigen tests for coccidioidomycosis are also available commercially.

The serum cryptococcal antigen is less likely to be positive in localized cryptococcal pneumonia compared with disseminated cryptococcosis. However, antigen detection is useful to confirm the diagnosis because the assay has excellent specificity; serial titers are not helpful in monitoring response to therapy. Positive blood cultures for *C. neoformans* are also more common when CNS infection is present. All patients with positive cryptococcal testing or suspected disease should undergo CSF testing to assess for CNS disease.

Gram stain and bacterial cultures of expectorated sputum may be helpful for patients suspected of having bacterial pneumonia. In addition, blood cultures should be obtained because *S. pneumoniae* bacteremia is seen more commonly in HIV-infected patients with pneumococcal pneumonia than in immunocompetent patients. Urinary antigens for *S. pneumoniae* and *L. pneumophila* may also be helpful. The diagnosis of TB is identical to the methods used in immunocompetent hosts. Evidence of prior exposure to TB (i.e., LTBI) can be assessed by TST or IGRA. If positive results are obtained or the patient has suggestive symptoms, active disease must be ruled out; TSTs and IGRAs are not sufficiently sensitive for this purpose. Assessing patients for active pulmonary disease involves obtaining sputum samples for AFB smear and culture and *M. tuberculosis* NAAT in patients whose AFB smear is positive or whose suspicion of disease is high.

Treatment

First-line therapy for PCP is trimethoprim-sulfamethoxazole with adjunctive steroids in patients with moderate to severe disease (as evidenced by the degree of hypoxemia) to counter

the innate immune response. Many patients with moderate to severe disease require mechanical ventilatory support during therapy, and mortality remains relatively high. Once treatment has been completed, antimicrobial prophylaxis (preferably with lower-dose trimethoprim-sulfamethoxazole) should be continued until persistent reconstitution of the immune system occurs, as demonstrated by an increasing CD4 cell count greater than 200/mm³ for at least 3 months.

Pulmonary cryptococcal and coccidioidomycosis infections can be treated with fluconazole in mild to moderate cases. For histoplasmosis, itraconazole is the therapy of choice. In severe disease with any of these fungal pathogens, lipid amphotericin B preparations are used in the initial phases of treatment, with transition to oral azole therapy in later stages once improvement is noted. The duration of therapy for these infections depends, in part, on whether the immune system is able to recover, similarly to PCP. However, in cases of diffuse pulmonary or disseminated coccidioidomycosis, life-long treatment is often recommended because of the high risk of relapse.

Treatment of active TB involves the same agents as for non-HIV-infected individuals, with the exception that drug-drug interactions between anti-TB medications and ART may necessitate slight modifications in TB or HIV regimens, dose adjustments, and close monitoring for toxicity. Because of the high risk of progression to active TB, HIV-infected patients with evidence of LTBI should be offered therapy to prevent progression to active disease.

HAART is the most important strategy for preventing opportunistic infections, especially because many of these pathogens are ubiquitous in the environment, and it is impossible to avoid exposure. One goal of HAART is to maintain the host immune response, evidenced by the CD4 cell count, at a level that reduces susceptibility to these opportunistic infections. Current data suggest that early initiation of HAART is beneficial in most cases and should be considered. Prophylaxis for opportunistic infections is often provided when the CD4 cell count decreases below certain levels. Other preventive measures for respiratory infections in patients with HIV infection include administration of pneumococcal and annual influenza vaccines.

Patients with other immunocompromised states

Case study

A 29-year-old male patient with a history of chronic myelogenous leukemia (CML) treated with hematopoietic cell transplantation presented to the oncology clinic with a 3-day history of worsening fatigue and cough with yellow-green sputum, but no fever. A chest CT scan showed pulmonary micronodules and focal consolidation, with necrosis and abscess formation within the areas of consolidation. The consolidation had worsened compared with a previous CT scan performed 1 month earlier. The patient had been taking immunosuppressive medications for hematopoietic cell transplantation.

Infections in immunocompromised patients depend on the interplay of several factors, such as the specific deficiencies in

immune system function, particular exposures of the patient, and, in the case of transplantation, the donor, and measures taken to mitigate infection risk, including antimicrobial prophylaxis. Neutrophils, or granulocytes, are an important component of the innate immune system. A decrease in absolute number below $500/\text{mm}^3$ (neutropenia), as seen with hematologic malignancies and after chemotherapy (including preparatory regimens before hematopoietic cell transplantation), or a defect in neutrophil function, as seen with some congenital disorders (e.g., chronic granulomatous disease), can result in increased susceptibility to infection. Impairments of the adaptive immune system include decreased B-cell function (humoral immunity) and decreased T-cell function (cell-mediated immunity). These are seen as a consequence of myelosuppressive and myeloablative chemotherapy, with immunosuppressive medications used post-hematopoietic cell and solid organ transplantation, and with certain congenital disorders. Exposures to infectious agents can occur in the community before hospitalization and transplantation and place the patient at risk for reactivation disease (e.g., *M. tuberculosis*, CMV), be transmitted in a donor organ at the time of transplantation (e.g., *Aspergillus* colonization in lung transplantation), or be acquired from the hospital setting (hospital-acquired infection).

Epidemiology

Patients with compromised immunity are at risk for all of the typical infections an immunocompetent person might acquire as well as opportunistic pathogens that are not clinically significant in the presence of an intact immune system. Neutropenia and defects in neutrophil function predispose patients to infections with bacteria, such as *S. aureus* and *P. aeruginosa*, and fungi, including *Aspergillus*, *Mucorales*, and *Candida*. These are most often seen in patients receiving chemotherapy and in patients in the immediate post-hematopoietic cell transplantation period; these patients often present with fever while neutropenic.

Case check 32.10

The patient with CML who has had hematopoietic stem cell transplantation is at risk not only for virulent infections that affect the immunocompetent population but also for development of opportunistic infections that require compromised host defenses to cause disease. His presentation with consolidating and necrotic micronodules that have slowly progressed over time is worrisome for an invasive fungal infection such as aspergillosis. Any conditioning regimen the patient received before transplantation (if recent), any recent neutropenia, and his chronic steroid use all put him at increased risk for invasive aspergillosis, which generally does not cause significant disease other than in patients who are immunocompromised or have chronic underlying lung disease. Given the delay in making a definitive diagnosis with a fungal culture and pathology and the patient's immunocompromised status, empiric antifungal therapy is warranted.

Defects in humoral immunity, such as hypogammaglobulinemia, result in recurrent respiratory infections in patients with congenital disorders, including an increased risk of infection with encapsulated organisms such as *S. pneumoniae* and *H. influenzae* and viral infections in patients recovering

from myeloablative therapy or receiving immunosuppressive medication.

Patients with acquired cell-mediated immunity defects, such as those seen with HIV infection or solid organ transplantation, take the longest time to recover, leaving them at risk for a myriad of infections, including mycobacterial infections such as TB; fungal infections including *P. jiroveci* and *H. capsulatum*; bacterial infections such as *Legionella* spp., *S. aureus*, and *Nocardia* spp.; and viral infections including CMV and other herpesviruses. Recovery of adaptive immunity can be significantly delayed in patients with chronic graft-versus-host disease (GVHD) and patients with graft rejection, both of whom generally require further immunosuppressive therapy in addition to the maintenance immunosuppression regimen they are already receiving.

Infections within the first month after transplantation are generally hospital acquired in origin with occasional donor-derived infections in this period. Immunosuppression is most intense within the first 12 months after transplantation, and opportunistic infections predominate during this time; prophylaxis can delay many of these infections, which may subsequently develop within a few months after stopping therapy. Later infections are more likely to be the same community-acquired infections that affect immunocompetent hosts.

Clinical manifestations

In general, immunocompromised hosts may not present with the same symptoms as immunocompetent individuals; manifestations of infections may be muted or atypical, necessitating a high index of suspicion. Infections with encapsulated organisms, such as pneumococcal pneumonia, are more common and may be more severe than in immunocompetent hosts. Given frequent exposure to broad-spectrum antimicrobials, long stays in the hospital, and other comorbidities, antimicrobial-resistant bacteria, including Enterobacterales, *Staphylococcus*, and *Pseudomonas*, are seen more commonly in hospital-associated cases. *Nocardia* spp. often cause disseminated infection in immunocompromised hosts, affecting the lung, brain, and skin.

Invasive *Aspergillus* infections usually involve the lower respiratory tract but can also affect the sinuses and skin. *Aspergillus* spp. can cause infection at the tracheal anastomosis after lung transplantation or pulmonary parenchymal disease in neutropenic leukemia patients. The risk of this infection is increased with neutropenia lasting longer than 2 weeks, corticosteroid use, GVHD, and fungal colonization of lung transplants. Pulmonary PCP in transplant patients can often have a more fulminant course with increased morbidity and mortality compared with that in HIV-infected patients. PCP has become increasingly less common with the use of routine prophylaxis after transplantation and with prolonged high-dose steroid use. In addition, many long-term chronic immunosuppressive regimens are now designed to be steroid sparing, further decreasing the risk.

Like aspergillosis, mucormycosis primarily affects the sinuses (rhinocerebral disease) and respiratory tract in susceptible individuals. Patients may present with signs and symptoms typical of pneumonia such as fever, cough with occasional hemoptysis, and dyspnea, but the course is often rapidly progressive. For cryptococcal pulmonary infection, the lung is the usual portal of entry for disseminated infection,

which frequently involves the CNS and causes fever, headache, and meningeal symptoms. As discussed earlier in the "Tuberculosis and Other Chronic Infections" section, the endemic mycoses may demonstrate an increased severity of disease in immunocompromised individuals.

Viral respiratory infections are common in all stages of the posttransplantation period. RSV, influenza virus, parainfluenza virus, adenovirus, and hMPV are commonly detected. CMV is among the most important causes of viral infection in transplant patients, and although some infections are asymptomatic, others can be severe. CMV syndrome manifests as fever in the presence of viremia. Symptoms of CMV tissue-invasive disease depend on the organ affected. Pneumonia can present as fever and dyspnea, whereas diarrhea and abdominal pain or vision loss may indicate involvement of the GI tract and retina, respectively.

As is the case with HIV-infected individuals, LTBI in the recipient can reactivate during the posttransplantation period; donor-derived infection or new infection caused by posttransplant exposure can also occur. Symptoms are dependent on the extent of disease. The possibility of LTBI should be assessed and treated when detected before transplantation, whenever possible.

The parasitic helminth *Strongyloides stercoralis* can cause life-threatening infection in immunocompromised hosts. The parasite is most prevalent in tropical and subtropical climates. In the United States, the southeastern states have the highest risk. Once acquired, *S. stercoralis* can remain dormant in the GI tract for decades. In patients receiving steroids and in transplant recipients, larvae can penetrate the bowel wall and invade surrounding tissues, causing a hemorrhagic enterocolitis and pneumonitis. Dissemination of the larvae is frequently accompanied by bloodstream invasion of gram-negative enteric bacteria and meningitis.

Patients who are profoundly neutropenic after chemotherapy commonly develop fevers during this period. Infection is documented in only about 20% to 30% of cases, although most patients receive empiric therapy, and infection may not be diagnosed in additional patients because of the limitations of diagnostic testing.

Laboratory diagnosis

The approach to the diagnosis of opportunistic respiratory tract infections in this patient population is similar to that used for patients with HIV and, in many cases, immunocompetent hosts. The same general principle also applies regarding the reduced ability to rely on parameters of host immune response (i.e., antibody production and inflammatory reactions) and the increased emphasis on histopathology and culture identification for diagnosis. Differentiating GVHD and graft rejection from infection is also critical. Because the differential diagnosis is so much larger for immunocompromised individuals, identifying a pathogen is even more critical, and the testing approach is often appropriately comprehensive.

Viremia, including for CMV, is generally assessed using quantitative serum PCR testing; tissue-invasive disease generally requires histologic evidence of the pathogen, however, as a positive PCR from the lung, for example, may represent viral shedding caused by acute illness, and a negative serum

PCR does not rule out tissue-invasive disease. In some cases, the diagnosis is empiric.

Treatment

Treatment of these infections is identical to that of similar infections in immunocompetent or HIV-infected patients in most cases, but a few differences warrant discussion. In general, immunocompromised patients may become worse much more quickly compared with immunocompetent hosts; therefore empiric therapy is generally warranted (e.g., for febrile neutropenia) while the diagnostic workup is in progress. Antimicrobial resistance may also be more of a concern in this patient population, both with bacterial and viral pathogens, and risk factors for drug resistance should be sought when considering an empiric regimen. It is also critically important to be aware of potential drug interactions because many antimicrobials can interact with immunosuppressive medications, placing patients at risk for increased toxicity or rejection of the graft, depending on the specific interaction. In patients receiving long-term immunosuppression for transplants, the occurrence of certain infections may indicate a state of overimmunosuppression, and when possible to accomplish without sacrificing graft survival, reduction in the intensity of immunosuppression is an important therapeutic tool.

Prophylactic antimicrobials to prevent PCP and other fungal infections and CMV and other herpesvirus infections are often used when chemotherapy is given or when transplantation is performed to reduce the incidence of these infections in the early posttransplantation period. Trimethoprim-sulfamethoxazole prophylaxis, which is commonly used to prevent PCP, is also effective for *Nocardia*, although some breakthrough infections do occur. For CMV, most transplantation centers use universal antiviral prophylaxis after transplantation or monitor patients with serial serum CMV antigen or PCR assays and preemptively begin treatment with the first signs of viremia. As is true for HIV-infected patients, vaccination also represents an important tool for infection prevention in these groups, including for *S. pneumoniae* infection and influenza.

Infection control measures become increasingly important when dealing with immunocompromised patients, who are at increased risk for complications and death. Because most respiratory pathogens in hematopoietic cell transplantation are acquired via inhalation, patients are often placed in what is termed a *protective environment room*, which has an increased number of air changes per hour, positive-pressure air flow, and high-efficiency particulate air (HEPA) filtration to remove some of these pathogens from inspired air. Adherence to handwashing and contact precautions will also help reduce the transmission of pathogens from health care workers and among patients.

Bioterrorism and respiratory infections

Since the anthrax attack in the United States in 2001, the threat of biological agents as weapons to cause mass death has become an important national security and public health issue. The CDC has labeled anthrax, plague, and tularemia as category A bacterial agents, which constitutes the highest

level of threat (see [Chapter 30](#)). All of these organisms can be aerosolized for delivery to the respiratory tract. The initial clinical presentation of these pulmonary illnesses is similar to that observed with common bacterial respiratory pathogens, but the subsequent course can be fulminant and rapidly fatal. Therefore the greatest diagnostic challenge is clinical suspicion of these rare pathogens in the differential diagnosis of pneumonia and the related use of appropriate diagnostic studies or resources. It is important for the clinician to discuss the possibility of these agents with the laboratory because infection can be transmitted to laboratory personnel with manipulation of the culture if it is not handled properly.

Inhalational anthrax caused by *Bacillus anthracis* has an incubation period of 1 to 7 days. Patients may initially present with fever, but subsequently develop rapidly progressive pneumonia and multisystem organ failure. Blood and sputum cultures are often positive for gram-positive bacilli that are nonhemolytic, nonmotile, and catalase positive. Such isolates are referred to reference laboratories in the Laboratory Response Network (LRN), where identification can be confirmed by molecular diagnostic testing, such as PCR assay. Anthrax lethal factor toxin in plasma and serology should also be pursued. Inhalational anthrax is not communicable from person to person.

Pneumonic plague is caused by *Yersinia pestis*. Plague is endemic in some areas of the western United States and other parts of the world where rodents serve as a reservoir. Pneumonic plague represents the least common form of endemic cases but should be expected in the event of bioterrorism-related aerosol release, in which the organism is inhaled. A short incubation period of hours to days precedes the sudden onset of fever and rapid respiratory failure. Pneumonic plague can be spread from person to person, and droplet precautions should be applied in suspected cases. Sputum, tissue, and blood cultures from infected individuals may show plump, gram-negative bacilli that can exhibit bipolar staining (closed safety pin–like appearance) with Giemsa or Wright stains; the organism grows well in culture. Confirmatory laboratory testing should be done through the LRN under strict biosafety conditions because of the risk of laboratory transmission.

Tularemia is a zoonotic illness caused by an aerobic gram-negative coccobacillus, *Francisella tularensis*. Pulmonary tularemia has a high mortality rate and, similarly to the agents mentioned, has a rapid onset of symptoms and progression to respiratory failure. Tularemia is not spread from person to person. These organisms require stringent growth conditions and are therefore difficult to isolate in culture. Specimens should be referred to an LRN facility, where a presumptive diagnosis may be made using DFA, PCR, and culture testing; serology is also indicated.

POINTS TO REMEMBER

- The method and site of collection, quality of the clinical specimen, and clinical context are all important factors to consider when distinguishing between colonization and infection.
- The age and immune status of the host will help determine likely infectious pathogens.
- The normal microbiota and specific anatomic structures play a role in defending the host from respiratory tract infection.
- Virulence factors of disease-producing organisms enable them to evade host defense mechanisms, cause clinical infection, and induce tissue disease.
- The prevalence of different viral respiratory pathogens varies according to seasonal changes in epidemiology.
- Newer pathogens such as SARS-CoV, MERS-CoV, SARS-CoV-2 can cause severe and sometimes fatal illness. It is important to be aware of their existence and clinical significance.
- Healthcare-associated pathogens are often resistant to multiple antimicrobial agents, and it is important to have a working knowledge of local antimicrobial resistance patterns.
- The occurrence of opportunistic disease in patients with HIV infection is a function of underlying host immunodeficiency and pathogen virulence.
- Immunocompromised patients, including those who have undergone hematopoietic cell or organ transplantation, are at increased risk for serious pneumonias caused by a wider range of pathogens (opportunistic agents) than that observed with immunocompetent patients.
- Awareness of potential agents of bioterrorism, as listed by the CDC, is important. Consider these uncommon pathogens on the list of possible causes in the differential diagnosis.

LEARNING ASSESSMENT QUESTIONS

1. Why is it important to distinguish between normal microbial biota and pathogenic microorganisms?
2. Why is it important to assess the immune status of the host when determining the importance of a microorganism detected in the diagnostic microbiology laboratory?
3. What is meant by an *opportunistic pathogen*?
4. Why should the microbiologist maintain awareness of seasonal trends in respiratory tract infections? Give examples of infectious agents for which these trends are noted.
5. How does the transmission of SARS-CoV-2 differ from other respiratory viruses?
6. What are common bacterial pathogens that cause CAP?
7. What is the epidemiologic importance of the distinction between antigenic drift and antigenic shift for influenza virus infections?
8. What is the difference between CAP and HAP as related to the likely bacterial pathogens? Why is there a different spectrum of pathogens for these two clinical situations?
9. What is the significance of the absolute CD4 cell count in determining the susceptibility to infection and the likely pathogens in a patient with HIV or AIDS?
10. What diagnosis should be suspected in a patient with a chronic pneumonia who is homeless and presents with a 3- to 4-week history of increasing fatigue, weight loss, and night sweats?
11. Which bacterial pathogens cause pneumonia and are designated as the highest-level threat (category A) potential agents of bioterrorism by the Centers for Disease Control and Prevention (CDC)?
12. What is the difference in laboratory protocol for diagnostic studies that should be considered for all agents of bioterrorism?

13. What should a microbiologist do if he or she finds α -hemolytic colonies on a properly collected sputum specimen for a patient suspected of having lobar pneumonia?
 - a. Ignore the colonies because they are normal microbiota.
 - b. Ignore the colonies because no known pathogens for pneumonia are α -hemolytic.
 - c. Do a full workup to identify the organism.
 - d. Suspect a bioterror agent and send the isolate to a laboratory response network reference laboratory.
14. What is the cause of reduced clearance of respiratory secretions that predisposes people to respiratory infections?
 - a. Obstruction by a foreign body
 - b. Alterations in the viscosity of the mucus
 - c. Immature anatomic development
 - d. All of the above
15. Which organisms are most frequently isolated from middle ear cultures of individuals with otitis media?
 - a. *Staphylococcus aureus* and *Escherichia coli*
 - b. *Streptococcus pneumoniae* and *Haemophilus influenzae*
 - c. *Streptococcus pneumoniae* and *Mycoplasma pneumoniae*
 - d. *Haemophilus influenzae* and *Neisseria meningitidis*
16. Which organism is the most common opportunistic pathogen that routinely infects patients with HIV/AIDS?
 - a. *Pneumocystis jirovecii*
 - b. *Mycoplasma pneumoniae*
 - c. *Serratia marcescens*
 - d. Respiratory syncytial virus
17. A 3-month-old infant presented with persistent symptoms that include paroxysmal cough, breathing difficulty, fever, and vomiting after a spasm of coughing. Cyanosis and intercostal retractions were noted. The infant also had a fever. Nasopharyngeal secretions were collected and analyzed. The organism was biochemically inert. Based on these clinical findings, what is the most likely suspected agent?
 - a. *Mycoplasma pneumoniae*
 - b. *Pneumocystis jirovecii*
 - c. *Bordetella pertussis*
 - d. Respiratory syncytial virus
18. A 25-year-old previously healthy female patient presented with sore throat, earache, and nonproductive cough of several days' duration. She had a low-grade fever and malaise, and although she did not feel completely well, she did not feel sick enough to stay in bed. Testing showed a normal white blood cell count with routine bacterial sputum culture negative for typical pathogens, but x-ray examination revealed pulmonary infiltrates. Based on these clinical findings, what is the most likely suspected agent?
 - a. *Mycoplasma pneumoniae*
 - b. *Pneumocystis jirovecii*
 - c. *Bordetella pertussis*
 - d. *Streptococcus pneumoniae*
19. A 67-year-old male patient who smokes 15 to 20 cigarettes per day presented with a persistent cough, malaise, and a fever of 102° to 105°F. On admission, the patient was disoriented and complained of nausea and vomiting. Repeat sputum cultures yielded only normal respiratory tract biota, although a direct Gram-stained smear showed 3+ PMNs and rare epithelial cells. Based on these clinical findings, what is the most likely suspected agent?
 - a. *Mycoplasma pneumoniae*
 - b. *Pneumocystis jirovecii*
 - c. *Legionella pneumophila*
 - d. *Streptococcus pneumoniae*
20. You received a sputum sample from a patient with history of chronic obstructive pulmonary disease. On admission, the direct Gram-stained smear showed gram-positive diplococci, more than 10 squamous epithelial cells per low-power field, and less than 25 polymorphonuclear cells per low-power field. Which of the following would be your interpretation of the direct Gram-stained smear?
 - a. The sample is acceptable for culture.
 - b. The sample is not acceptable for culture.
 - c. The number of squamous epithelial cells present in sputum are acceptable.
 - d. The gram-positive diplococci present are significant if recovered in culture.

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Skin and soft tissue infections

Nina M. Clark

CHAPTER OUTLINE

Anatomy of the skin, 833

Skin microbiota, 833

Localized bacterial and fungal skin infections, 834

Dermatitis, 834

Pyoderma, 837

Other soft tissue infections, 841

Nodular lymphangitis, 845

Dermatologic manifestations of systemic bacterial and fungal infections, 847

Bacteria, 847

Fungi, 852

Viral infections, 853

Rubeola, 853

Rubella, 853

Parvovirus B19 infection, 854

Enteroviral infections, 854

Herpesviridae, 854

Molluscum contagiosum, 856

Orf and Milker nodule, 857

Human papillomavirus, 857

Alphaviruses, 857

Hemorrhagic fever viruses, 858

Severe acute respiratory syndrome–coronavirus-2 (SARS-CoV-2), 858

Parasitic infections, 858

Helminths, 859

Leishmaniasis, 860

Ectoparasites, 861

Immune- or toxin-mediated dermatologic manifestations of infectious agents, 861

Immune-mediated cutaneous disease, 861

Toxin-mediated cutaneous disease, 863

Laboratory diagnosis, 864

Bibliography, 866

OBJECTIVES

After reading and studying this chapter, you should be able to:

1. Describe the function of the skin as a host defense mechanism.
2. Identify the organisms that constitute normal skin biota.
3. Contrast the clinical presentations and the most common causative agents of each of the following types of skin infections: dermatitis, folliculitis, furuncle, carbuncle, impetigo, erysipelas, and cellulitis.
4. Differentiate risk factors, causative agents, and clinical manifestations of the following soft tissue infections: diabetic foot infections, infectious gangrene, mycetoma, and nodular lymphangitis.
5. Recognize the dermatologic manifestations of systemic bacterial infections.
6. Compare the dermatologic manifestations of fungal infections including systemic candidiasis.
7. Identify the etiologies and describe the manifestations of the common childhood viral exanthems.
8. Describe the pathogenesis of herpesvirus skin diseases.
9. Categorize the most frequent parasitic causes of cutaneous infection and describe their typical appearances.
10. Describe the characteristics and usual causative agents associated with disseminated intravascular coagulation, vasculitis, toxic shock syndrome, scarlet fever, and immune complex and embolic disease.
11. Evaluate the strengths and weaknesses of the common laboratory methods for diagnosing skin infections.

KEY TERMS

Bullae

Carbuncle

Cellulitis

Chromoblastomycosis

Colonization

Dermatophytosis

Ecthyma gangrenosum

Ectoparasite

Entomophthoromycosis

Erysipelas

Erysipeloid

Erythrasma

Exanthem

Folliculitis

Furuncle

Gas gangrene

Hidradenitis suppurativa
Impetigo
Intertrigo
Leprosy **Lobomycosis**
Methicillin-resistant
Staphylococcus aureus
(MRSA)
Molluscum contagiosum
Mycetoma
Nodular lymphangitis
Necrotizing fasciitis
Orf virus

Paronychia
Petechiae
Phaeohyphomycosis
Purpura fulminans
Pyoderma
Scarlet fever
Tinea
Toxic shock syndrome
Vasculitis
Vesicle
Zoonoses

Case in point

A 60-year-old male patient with poorly controlled type 2 diabetes presented to the emergency department with high-grade fever, chills, and severe pain and swelling in his right foot and lower leg. Two days before admission, he was working outside his home and scraped his lower leg against a brick wall. This area became red, swollen, and progressively more painful over 48 hours. On physical examination, the patient was febrile (103° F, reference range 97° to 99° F), his blood pressure was low (90/50 mm Hg, reference range 120/80 mm Hg), and the right leg and foot were red, swollen, and markedly tender, with several purple bullae starting to develop. His white blood cell count was elevated ($22 \times 10^9/L$, reference range 4.5 to $11 \times 10^9/L$). Blood cultures were obtained, and intravenous antimicrobial therapy was started. A computed tomography scan of the leg showed gas formation in the subcutaneous tissues. The patient was taken to surgery and found to have necrosis of the subcutaneous tissues down to the fascia, with purulent material present. The area was extensively debrided, and tissue and pus were sent to the laboratory for Gram stain and culture. These specimens grew multiple organisms, including *Escherichia coli*, *Bacteroides* sp., and *Peptostreptococcus* sp. The blood cultures also grew *E. coli*. After several additional debridements, a long hospitalization, and skin grafting, the patient fully recovered.

Issues to consider

After reading the patient's case history, consider:

- The clinical clues (e.g., patient characteristics, information obtained from the history and physical examination) used by the health care provider to diagnosis the patient's illness
- The specimen collection procedures appropriate to maximize recovery of the infectious agents
- The sample processing that should occur once the specimen arrives in the laboratory
- The types of organisms likely to be recovered on culture based on the site of infection and the events leading to the infection

The skin is the body's first line of defense against invasion by pathogens or by bacteria and fungi that inhabit the skin surface. As a dynamic physical barrier, the skin continually undergoes epithelial cell turnover, removing substances as well as potentially pathogenic microorganisms on its surface. In addition, the skin is colonized with a variety of resident microbes that perform a protective function.

This chapter discusses the following:

- The role of indigenous skin biota and other organisms in the pathogenesis of skin infection
- The clinical features and causes of various primary skin and soft tissue infections
- The dermatologic manifestations of systemic infections
- The diagnosis and treatment of specific types of skin and soft tissue infections

Anatomy of the skin

The skin consists of three layers: the epidermis, dermis, and subcutaneous layer (Fig. 33.1). The epidermis is the outermost layer and is composed of several layers of epithelial cells. The stratum corneum, the outermost layer of the epidermis, contains dead cells consisting of a protein called *keratin*. The second skin layer, the dermis, is a thick layer composed of connective tissue. Sweat gland ducts, hair follicles, and oil gland ducts are found in the dermis and penetrate the subcutaneous layer. These structures also provide potential passageways through which microbes can enter the skin. Sebum and perspiration provide moisture and nutrients necessary for the growth of certain microbes. Salt and lysozymes contained in perspiration and fatty acids found in sebum can inhibit the proliferation of some pathogenic microorganisms.

Skin microbiota

Even though it is not a particularly inviting habitat for microbes, the skin is continually exposed to the environment and is inhabited by millions of microorganisms. The usual biota of the skin can adapt to its high salt concentration, exposure to the elements, and relative lack of nutrients and moisture. The normal resident skin microbiota is made up of an array of bacteria, fungi, and viruses that are adjacent to the epidermis but can also be in the dermal tissues. The organisms can act as competitive inhibitors of pathogens but breaks in the skin may allow normal biota to cause infection and permit more transient organisms to enter and cause disease.

Variations in the skin microbiota from person to person are based on a number of factors such as gender, age, geography, hygiene, and the presence of skin or other diseases. Skin biota also vary in body location depending on changes in temperature, moisture, and concentration of sebaceous or sweat glands. However, important members include gram-positive cocci, particularly staphylococci and streptococci; coagulase-negative staphylococci such as *Staphylococcus epidermidis* are permanent skin residents, while coagulase-positive *Staphylococcus aureus* is a transient colonizer. Other members of the normal biota include the gram-positive bacilli *Cutibacterium acnes* and *Corynebacterium* and yeasts *Candida* and *Malassezia*. These resident organisms may become pathogens if there is trauma to the skin. Although vigorous washing reduces the amount of surface skin biota, it does not eliminate resident microbiota **colonization**. The acidic pH of skin (~5) inhibits many common skin pathogens.

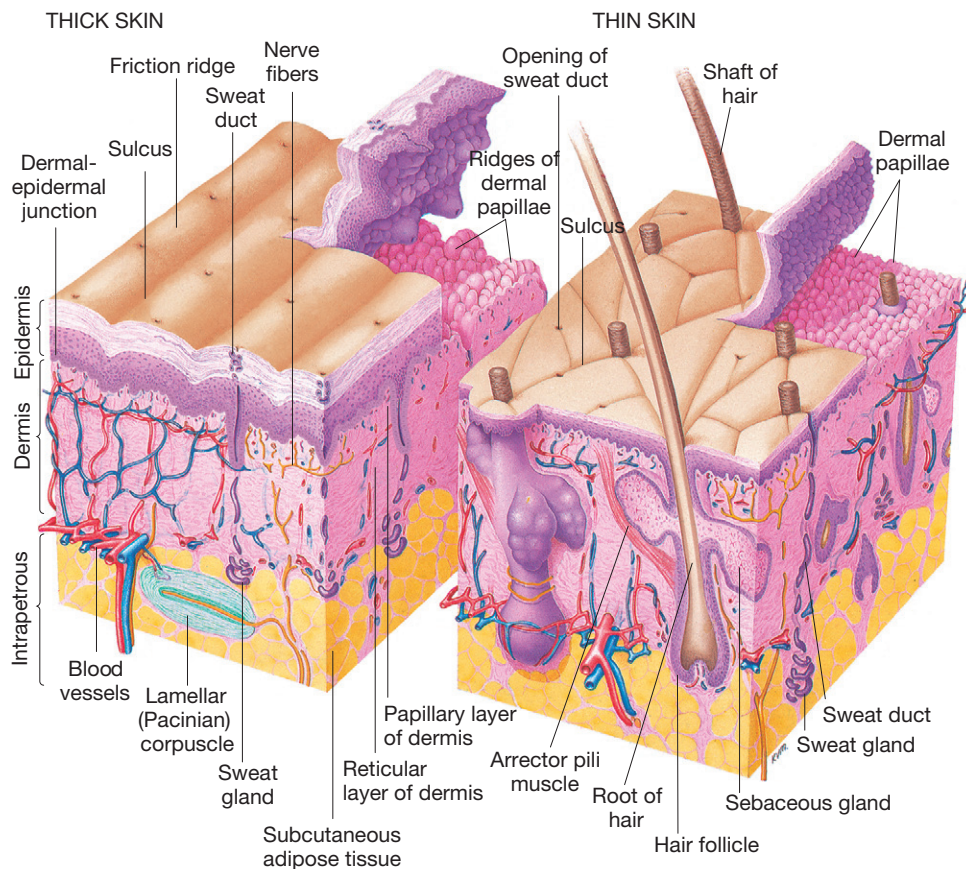


Fig. 33.1 Anatomy of the skin. (From Patton, K. T., & Thibodeau G. A. [2019]. *Anatomy and physiology* [10th ed.]. St Louis: Elsevier.)

Localized bacterial and fungal skin infections

An extensive list of infections can involve the skin (Box 33.1). Bacteria cause the most skin infections, and they have become more difficult to treat because of increasing antimicrobial resistance. Table 33.1 shows the results of a survey of the causative agents of skin and soft tissue infections isolated from patients in North America between 1998 and 2004. *S. aureus*, an organism that is resistant to many common antimicrobials, was the predominant skin and soft tissue pathogen, accounting for almost 45% of over 5800 isolates. *Pseudomonas aeruginosa*, another microorganism that displays multidrug resistance, ranked a distant second at 11%.

Skin and soft tissue infections may be classified in multiple ways. One method classifies them based on the appearance of the skin lesion, which provides an important clue about possible causative organisms, as noted in Box 33.1. Skin and soft tissue infections also may be classified, as they are in this chapter, according to whether they occur as a manifestation of systemic disease or as a primary skin process. Primary skin infections can then be classified by the skin and soft tissue structure affected, and further subdivided by causative organism (e.g., bacterial, viral, mycobacterial, fungal, parasitic). It is also notable that a variety of the pathogens described in this chapter may cause infection as a secondary process, gaining

access to the skin after it is disrupted as a result of another preexisting skin condition (Table 33.2).

The first section of this chapter examines the clinically important and prevalent infections of skin and soft tissues that typically occur in localized areas of the body. They are presented in order from the most superficial infections to the deeper, more serious infections. They are predominantly the result of bacterial and fungal infection. Additional sections describe skin manifestations of systemic infections caused by bacteria, fungi, viruses, and parasites, including immune- or toxin-mediated dermatologic diseases triggered by pathogens.

Dermatitis

Dermatitis is a general term that describes an inflammation of the skin. It is characterized by areas of redness, swelling, and sometimes scaling of the skin and/or pruritus. There are many causes, only some of which are related to infection. The following section focuses on the most common infectious causes of dermatitis.

Intertrigo and superficial candidiasis

Intertrigo (intertriginous dermatitis) is an inflammatory cutaneous condition that occurs in body areas subjected to heat, moisture, and friction, which work together to cause skin breakdown. Infectious agents enhance this process.

BOX 33.1 Some infectious causes of skin lesions and their morphologic manifestations**Macular, papular, or maculopapular rashes**

Rubeola (measles)
 Rubella (German measles)
 Roseola
 Other viral exanthems
 Scarlet fever
 Toxic shock syndrome
 Secondary syphilis
 Severe acute respiratory syndrome–coronavirus-2 (SARS-CoV-2)

Smooth papules

Molluscum contagiosum
 Condyloma latum (secondary syphilis)

Verrucous papules or plaques

Condyloma acuminata (anogenital warts)
 Nongenital cutaneous warts
 Cutaneous tuberculosis
 Blastomycosis
 Coccidioidomycosis
 Chromomycosis
 Wheals
 Urticaria
 Scabies
 Cercarial dermatitis (swimmer's itch)
 Pruritic papules
 Scabies
 Folliculitis
 Erythema infectiosum (fifth disease)
 Bacterial cellulitis
 Necrotizing (gangrenous) cellulitis, fasciitis, myonecrosis
 Disseminated mycoses

Serpiginous or annular plaques

Erythema multiforme
 Erythema migrans (Lyme borreliosis)
 Cutaneous larval migrans (creeping eruption)

Vesicles or bullae

Herpes simplex virus
 Herpes zoster
 Varicella (chickenpox)
 Hand-foot-and-mouth disease

Herpangina
 Staphylococcus scalded-skin syndrome

Pustules

Folliculitis
 Impetigo
 Acne
 Disseminated gonococcal infection
 Furuncles, carbuncles
 Kerion
 Herpetic whitlow
 Ecthyma contagiosum (orf)
 Milker's nodule
 Hidradenitis suppurativa

Petechiae, purpura, and ecchymoses

Rocky Mountain spotted fever
 Other rickettsial infections
 Meningococemia
 Gonococemia
 Infective endocarditis
 Plague
 Dengue and other hemorrhagic fever viruses
 Enteroviral infections
 Leptospirosis
 Severe acute respiratory syndrome–coronavirus-2 (SARS-CoV-2)

Ulcers or necrosis

Primary syphilis
 Herpes simplex virus
 Chancroid
 Lymphogranuloma venereum
 Granuloma inguinale
 Impetigo
 Ecthyma gangrenosum
 Sporotrichosis
 Nontuberculous mycobacteria
 Nocardiosis
 Histoplasmosis
 Anthrax
 Ecthyma contagiosum (orf)
 Tularemia
 Leishmaniasis

Table 33.1 Order of bacteria causing skin and soft tissue infections in North America, 1998 to 2004

Rank	Pathogen	No. of isolates (% of total)
1	<i>Staphylococcus aureus</i>	2602 (44.6)
2	<i>Pseudomonas aeruginosa</i>	648 (11.1)
3	<i>Enterococcus</i> spp.	542 (9.3)
4	<i>Escherichia coli</i>	422 (7.2)
5	<i>Enterobacter</i> spp.	282 (4.8)
6	<i>Klebsiella</i> spp.	248 (4.2)
7	β -Hemolytic streptococci	237 (4.1)
8	<i>Proteus mirabilis</i>	166 (2.8)
9	Coagulase-negative staphylococci	161 (2.8)
10	<i>Serratia</i> spp.	125 (2.1)

From Moet, G. J., et al. (2007). Contemporary causes of skin and soft tissue infections in North America, Latin America, and Europe: report from the SENTRY Antimicrobial Surveillance Program (1998–2004). *Diagnostic Microbiology and Infectious Disease*, 57, 7.

Intertrigo usually occurs in the skin folds, typically the axillae, perineum, beneath the breasts, and in abdominal folds. Risk factors include obesity, incontinence, diabetes, compromised immunity, and extremes of age (e.g., diaper rash in infants). The most common organism present in these areas is *Candida*, although *S. aureus* and Enterobacterales also can play a role.

Intertrigo is one form of superficial candidiasis, but *Candida* can affect the skin and mucous membranes in other forms. Thrush is a type of candidiasis involving the oral mucosa characterized by white, curdlike patches on the tongue, palate, or buccal mucosa. These patches adhere to the mucosa but can be removed by scraping, leaving a raw, erythematous base. A similar process can occur in the vulvovaginal area, leading to *Candida* vaginitis. Balanitis, an inflammation of the glans penis that can spread to the thighs, scrotum, and buttocks, is commonly caused by *Candida* as well. Thrush and *Candida* vaginitis can be the result of antimicrobial use or immunocompromised state (e.g., due to human immunodeficiency infection or corticosteroid use). The balance of the normal microbiota can be altered by antimicrobial agents or immunosuppression and allow an overgrowth of yeast that

Table 33.2 Infections secondary to preexisting skin lesions

Infection	Major pathogen
Surgical wound infection	
Clean	<i>Staphylococcus aureus</i> , gram-negative bacilli
Contaminated, such as colon	<i>S. aureus</i> , streptococci, Enterobacterales, anaerobes
Intravenous catheter or percutaneous drain sites	<i>S. aureus</i> , coagulase-negative staphylococci, streptococci
Trauma	
Soil contamination	<i>Pseudomonas aeruginosa</i> , <i>Clostridium</i> spp., <i>Nocardia</i> spp., fungi
Freshwater contamination	<i>Aeromonas</i> , <i>Plesiomonas</i> , <i>Mycobacterium marinum</i>
Saltwater contamination	<i>Vibrio vulnificus</i> , <i>M. marinum</i>
Intravenous drug use	<i>S. aureus</i> , <i>Pseudomonas</i> spp., <i>Clostridium</i> spp.
Bites	
Human	Oral aerobes and anaerobes, <i>S. aureus</i>
Dog, cat	<i>Pasteurella multocida</i> , <i>S. aureus</i> , <i>Capnocytophaga canimorsus</i> , anaerobes
Rat	<i>Streptobacillus moniliformis</i> , <i>Spirillum minus</i>
Arthropod	<i>Yersinia pestis</i>
Other	
Decubitus ulcer	Streptococci, <i>S. aureus</i> , Enterobacterales, <i>Pseudomonas</i> spp., anaerobes, including <i>Bacteroides fragilis</i>
Foot ulcer in diabetic patients	<i>S. aureus</i> , streptococci, Enterobacterales, <i>P. aeruginosa</i> , anaerobes
Hidradenitis suppurativa	<i>S. aureus</i> , streptococci, Enterobacterales, <i>Pseudomonas</i> spp., anaerobes
Burns	<i>S. aureus</i> , <i>Candida</i> , <i>P. aeruginosa</i>

is normally present on skin or mucosal surfaces. *Candida* can also cause paronychia, an inflammation of the folds of the skin bordering the nail beds (see later), or onychomycosis, an infection of the nail itself.

Superficial forms of candidiasis are often treated successfully with topical antifungal agents such as nystatin or clotrimazole. Oral antifungal agents such as fluconazole, itraconazole, or voriconazole may be necessary for the treatment of more severe infections, particularly in those who are immunocompromised.

Erythrasma

Erythrasma is a superficial, chronic skin infection that manifests as pruritic, reddish-brown macules that are lightly scaled and wrinkled. The lesions are usually found in intertriginous areas, especially the groin, inner thighs, and toe webs. Axillae, intergluteal folds, and inframammary regions

are less often affected. The infection occurs more often in men, obese individuals, and patients with diabetes and is generally localized and benign but can become widespread in those with an impaired immune system. *Corynebacterium minutissimum*, a normal skin biota resident, is the causative organism and can be observed as filamentous rods with a Gram stain of samples taken from the stratum corneum. The lesions also produce a coral red fluorescence when examined under a Wood lamp. Topically administered erythromycin or clindamycin or orally administered erythromycin or clarithromycin are useful in the management of erythrasma. Topical 2% mupirocin has also shown efficacy in recent reports.

Dermatophytoses

For most dermatophytoses, humans are the primary reservoir. Occasionally, infections are acquired from infected domestic animals or soil. Dermatophytes are molds that colonize only keratinized surfaces of the body, including hair, nails, and skin. These organisms can invade the stratum corneum of the skin, causing superficial infection in various regions of the body; the feet, groin, scalp, and nails are affected most often. Dermatophytes within four genera (*Trichophyton*, *Epidermophyton*, *Nannizzia*, and *Microsporum*) generally cause infection. The mode of transmission is through direct skin-to-skin contact or indirect contact via contaminated fomites or environmental surfaces. However, it seems that all persons are not equally susceptible to infection.

The classic lesion of a dermatophyte infection is a circular scaly patch of erythema with a raised border (Fig. 33.2). The edges are often more inflamed than the center. These infections are also known as *ringworm*, reflecting the tendency of some lesions to expand in a circular fashion. The term **tinea**, used to denote a dermatophyte infection, is attached to a descriptor of the site of infection. For example, tinea cruris (jock itch) is a dermatophyte infection of the groin and perianal region, tinea pedis (athlete's foot) involves the feet, and tinea capitis is infection of the scalp (Fig. 33.3). In addition to *Candida*, dermatophytes are another cause of onychomycosis. *Tinea unguium* is the term used for this infection, which can result in significant thickening and discoloration of the nails. Tinea corporis (see Fig. 33.2) is a dermatophyte infection of the body in general, and often involves the trunk and legs.

The diagnosis of dermatophytosis and the identification of its causative agents can generally be accomplished by taking scrapings of lesions, softening the material in a 10% to 20% potassium hydroxide solution, and then examining the specimens microscopically for fungal hyphae. Calcofluor is useful to stain hyphae but requires the use of a fluorescent microscope. Skin or nails can be scraped with a scalpel blade, or nails can be clipped to obtain material for identification. Scrapings can also be observed under a Wood lamp, where certain dermatophytes (*Microsporum*) will fluoresce yellow-green. The precise mycologic cause can be confirmed by culturing infected material on Sabouraud dextrose agar (SDA). Dermatophytosis can be treated with a wide variety of topical antifungal agents, most commonly azole medications such as miconazole, clotrimazole, or econazole. Nail infections often require oral systemic antifungal therapy for treatment (e.g., terbinafine, itraconazole, or fluconazole).

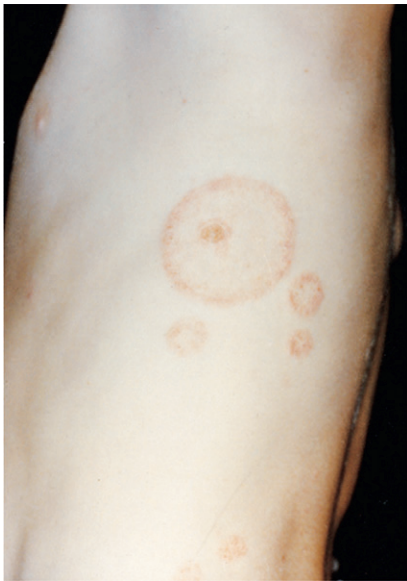


Fig. 33.2 Tinea corporis.



Fig. 33.3 Tinea capitis.

Tinea versicolor

Tinea versicolor, or pityriasis versicolor (Fig. 33.4), is an extremely common dermatitis that occurs worldwide. Unlike the tinea infections described earlier, tinea versicolor is not caused by a dermatophyte but by normal skin commensals. The lipophilic *Malassezia* spp. (*Malassezia globosa*, *M. sympodialis*, *M. furfur*) and other *Malassezia* spp. cause tinea versicolor. The characteristic skin manifestation of tinea versicolor is a diffuse distribution of hypopigmented or, less commonly, hyperpigmented macules, principally located on the trunk and proximal portions of the extremities. The lesions are usually nonpruritic and often coalesce to form scaly plaques.

The clinical features suggest the diagnosis, but it is recommended to confirm with a potassium hydroxide preparation

of skin scrapings because dermatophyte infections or *Candida* dermatitis could appear similar. *Malassezia* will display a “spaghetti and meatballs” appearance under the microscope, with round yeast forms and filamentous hyphae (see Chapter 27, Fig. 27.11). Spontaneous remission may occur in some patients; for others, topically administered azoles (e.g., ketoconazole) or topical terbinafine are effective. Selenium sulfide lotion can also be used.

Pyoderma

The **primary pyodermas** are a group of inflammatory skin disorders caused by bacteria that produce pus. The terms *impetigo* and *pyoderma* have been used interchangeably by some authors and in clinical practice, but there are other forms of pyoderma (see later), as listed in Table 33.3.

Impetigo

Impetigo is a common pyoderma that is most often seen in children (Fig. 33.5). Historically, most cases were caused by group A streptococci (GAS; *Streptococcus pyogenes*), although *S. aureus* has become the predominant pathogen over the past 20 years. The frequency of **methicillin-resistant *Staphylococcus aureus*** (MRSA) varies depending on the population studied and the presence of overcrowded living conditions or underlying diseases like diabetes mellitus. Group B *Streptococcus* occasionally causes impetigo in newborns secondary to the acquisition of colonizing vaginal microbiota from the mother. Impetigo is common in hot and humid climates and is highly contagious, particularly in households with poor hygiene. Initially, the lesions of impetigo begin as small **vesicles** that become pustules that rupture. The discharge is thick and yellow and dries to form the classic golden crusts. The lesions are superficial and painless and do not scar. They can be pruritic and are easily spread by scratching.

The bullous form of impetigo is much less common. It is caused by strains of *S. aureus* that produce exfoliative toxins leading to the formation of **bullae** that quickly rupture and form a transparent, light brown crust. The diagnosis of impetigo can be made from a Gram-stained smear and culture of the vesicular contents, and it is important to distinguish the lesions from herpes simplex virus (HSV), which can also cause vesicular or crusted skin lesions (see the “Herpes Simplex Viral Infection” section later in this chapter).

Orally administered penicillin is efficacious for streptococcal impetigo, but because many cases involve *S. aureus*, penicillinase-resistant oral penicillins such as dicloxacillin or an antistaphylococcal cephalosporin such as cephalexin are better options for empiric treatment. If the patient is allergic to penicillin, clarithromycin or erythromycin may be used. Because of the possibility of community-acquired MRSA infection (CA-MRSA; see the “Cellulitis” section later in this chapter), antimicrobials with activity against these organisms, such as doxycycline, clindamycin, or trimethoprim-sulfamethoxazole, may be preferable if a significant level of CA-MRSA is circulating in the community or if there is inadequate response to narrower-spectrum agents. If the infection is mild and the lesions are not numerous, topical rather than oral antimicrobial agents should be used, including 2% mupirocin or retapamulin. Mupirocin, unlike

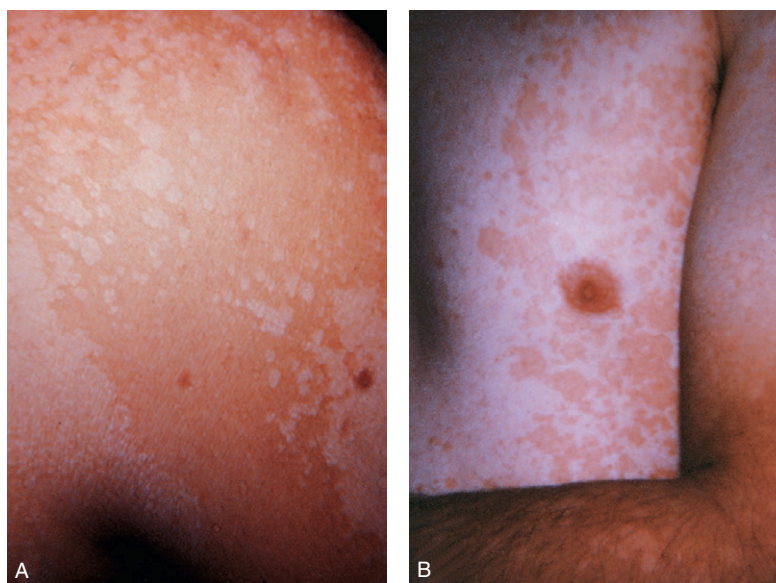


Fig. 33.4 Hypopigmented (A) and hyperpigmented (B) rash of tinea versicolor.

Table 33.3 Common primary pyodermas		
Infection	Organism	Features
Impetigo	<i>Staphylococcus aureus</i> , <i>Streptococcus pyogenes</i>	Children affected most; communicable; no fever
Erysipelas	<i>S. pyogenes</i> ; occasionally other β -hemolytic streptococci or <i>S. aureus</i>	Distinct raised borders; fever common
Cellulitis	<i>S. pyogenes</i> , <i>S. aureus</i> ; <i>Haemophilus influenzae</i> in children	Erythema, tenderness, pain, edema, warmth; fever common
Folliculitis	<i>S. aureus</i> ; gram-negative bacilli or <i>Candida</i> if predisposing conditions, <i>Malassezia</i> spp.	Papules around hair follicles; areas exposed to whirlpool bath (<i>Pseudomonas aeruginosa</i>)
Furuncle	<i>S. aureus</i>	Fluctuant, painful nodules often in intertriginous areas
Carbuncle	<i>S. aureus</i>	Multiple abscesses
Paronychia	<i>S. aureus</i> , <i>S. pyogenes</i> , gram-negative bacilli, <i>Candida</i>	Periungual swelling



Fig. 33.5 Bullous impetigo caused by *Staphylococcus aureus*.

retapamulin, is approved for topical treatment of MRSA, although resistance to mupirocin among MRSA strains has been increasing.

Erysipelas

Erysipelas is a type of superficial skin infection that involves not only the epidermis but also the underlying dermis and lymphatic system. Erysipelas is usually seen in children and older adults and is sometimes preceded by a respiratory β -hemolytic streptococcal infection. In the past, the infection

typically occurred on the face (Fig. 33.6), but now it is much more frequently seen in the lower extremities. Predisposing factors in adults include diabetes mellitus, alcohol abuse, venous stasis, trauma, skin ulcers or chronic inflammatory skin conditions, and lymphatic obstruction.

The lesions of erysipelas are characterized as painful, indurated areas of inflammation with raised borders that are sharply demarcated from the adjacent normal skin. The involved skin usually has a bright red to crimson hue, and patients often have fever. Most uncomplicated cases remain confined to the dermis and lymphatics, but deeper extension with cellulitis (see the “Cellulitis” section), abscess formation, or necrotizing fasciitis may occur, and bacteremia is found in a minority of cases, making erysipelas a potentially life-threatening infection.

Most infections are caused by GAS, with a minority of infections caused by other β -hemolytic streptococci and *S. aureus*. Usually, the diagnosis is based on the clinical presentation because it is difficult to isolate GAS from the skin lesions. On occasion, aspiration of the advancing edge of



Fig. 33.6 Erysipelas caused by *Streptococcus pyogenes*.

a lesion with culture of the aspirate has been successful in identifying the organism. Penicillin is the primary treatment, although first-generation cephalosporins such as cephalexin or erythromycin can be effective therapy.

Erysipeloid

Erysipeloid is a superficial soft tissue infection caused by *Erysipelothrix rhusiopathiae*, a gram-positive rod. It is an occupational hazard of handlers of animals, meat, poultry, hides, and saltwater fish. Because it is typically introduced by trauma, the fingers and dorsum of the hand are the most frequent sites of infection. Mimicking erysipelas, the erysipeloid lesion is red and painful, with raised borders. Septic arthritis and bacteremia may occur as complications. The organism is difficult to see on Gram stain but may be isolated in culture. Penicillin G is the preferred treatment.

Anthrax

Bacillus anthracis, the agent of anthrax, is a gram-positive rod that causes an acute febrile illness with a mortality rate ranging from <2% with cutaneous disease to 45% for pneumonia and 92% for anthrax meningitis. The cutaneous form of anthrax is an ulcerative disease and is more common than pulmonary or systemic infection. The skin lesions typically occur in persons working with infected animals (sheep, goats, cattle, and other herbivores) or animal products such as wool that are contaminated with *B. anthracis* spores. Intravenous (IV) drug use is another mechanism for infection, although anthrax is uncommon in the United States. A bioterrorism-related anthrax outbreak did occur in the United States in 2001 when *B. anthracis* spores were sent by mail to media and congressional offices resulting in 11 cutaneous and 11 pulmonary cases of anthrax and 5 deaths.

Anthrax skin lesions are usually seen on the face, neck, or arms at sites of minor abrasions. They begin as painless papules that vesiculate and are surrounded by significant erythema and a gelatinous type of edema. The vesicle evolves into a hemorrhagic and then necrotic lesion, and an eschar forms, although the skin lesions remain painless. Regional lymphadenopathy can be present, and bacteremia may occur with fever and hypotension. The diagnosis is often suspected

clinically given the characteristic skin lesions, but biopsy and culture should be performed for confirmation. The finding of *B. anthracis* from a clinical specimen constitutes a critical value and should be communicated to the patient's health care provider immediately.

Penicillin has long been the drug of choice for the treatment of anthrax, but with the bioterrorism cases that occurred in the United States, concerns have arisen that strains can be deliberately altered so they are resistant to commonly used antimicrobial agents. Therefore treatment with ciprofloxacin or doxycycline has been recommended as initial therapy. Anthrax vaccine is licensed for preexposure prophylaxis for adults at high risk for exposure to *B. anthracis* and, in combination with antibiotics, for adults with suspected or known exposure to aerosolized *B. anthracis* spores.

Cellulitis

Cellulitis is a diffuse inflammation and infection of the superficial skin layers and subcutaneous tissues. It is a very common form of skin and soft tissue infection that accounts for many hospitalizations and physician visits each year. Cellulitis extends deeper in the soft tissues than erysipelas and appears as an area of painful erythema, warmth, and edema of the skin, with poorly defined margins. Drainage of pus from areas of cellulitis may occur (purulent cellulitis), and these cases are usually caused by *S. aureus*. Depending on its extent and severity, cellulitis may or may not be accompanied by fever and other clinical or laboratory features of systemic infection (e.g., malaise, rigors, headache, elevated white blood cell count). Predisposing factors include surgery or other trauma and underlying skin disorders, such as ulcers or dermatitis.

Blood cultures are usually negative, and the diagnosis is often made clinically, based on the appearance of the affected area. In some cases, such as in immunocompromised patients or those who are not responding to empiric antimicrobial therapy, it may be useful to perform needle aspiration at the leading edge of an area of cellulitis for aerobic and anaerobic culture. Rates of organism recovery with this procedure have ranged in studies from 0% to 40%. The most common causative agents are GAS and *S. aureus*, although group B, C, and G streptococci can also cause cellulitis. Intravenous or oral systemic antimicrobial agents directed at *S. aureus* and streptococci are generally effective treatment for cellulitis. Patients who experience trauma or who have chronic open wounds, such as diabetic patients with foot ulcers, may develop surrounding cellulitis caused by a mixture of gram-positive and gram-negative organisms, including anaerobes (see later), and may require broader antimicrobial therapy.

Recurrent cellulitis

Lymphedema, obesity, venous stasis, and untreated tinea pedis are associated with an increased risk of developing recurrent skin and soft tissue infections. Breaks in skin integrity can allow the development of infection, and impaired lymphatic drainage can impede host immune responses and clearance of pathogens. In addition, bouts of infection can lead to scarring and further chronic edema. Patients with frequent cellulitis recurrences may be treated with long-term antimicrobial regimens in an attempt to decrease the rate of infection.

Methicillin-resistant *Staphylococcus aureus* infections

S. aureus is the most common cause of skin and soft tissue infections in the United States. Approximately 20% of individuals persistently carry *S. aureus* in the nares, and another 30% carry the organism intermittently. This carriage increases the risk that an individual will develop staphylococcal infection. *S. aureus* has acquired resistance to a variety of antimicrobials, and MRSA accounts for many cases of skin and soft tissue infection. MRSA infections are associated with higher mortality rates and greater health care costs compared with those caused by methicillin-susceptible *S. aureus*.

MRSA is prevalent in the hospital setting, where 25% to 50% of *S. aureus* isolates are methicillin resistant; the proportion is usually higher in intensive care units. However, MRSA has also increased in frequency in the community since the 1980s among persons with no prior exposure to hospitals, nursing homes, dialysis centers, or other health care settings. Investigations of CA-MRSA cases have shown that children, incarcerated persons, military personnel, athletes involved in contact sports, IV drug users, household contacts of persons with CA-MRSA infection, men who have sex with men, and Native Americans are at increased risk for infection by CA-MRSA. The extensive use of antimicrobials in livestock has also led to widespread carriage of MRSA in animals, and this can be a source of infection for humans. Several different strains of CA-MRSA are known, but strain USA300 is the most prevalent and has been associated with more severe disease, including bacteremia, necrotizing pneumonia, and severe soft tissue infection. Of note, this strain has also become prevalent in health care institutions. Although the incidence of hospital-onset MRSA infections has decreased, likely because of efforts in reducing healthcare-associated infections, progress against MRSA has seemed to stall since 2013.

MRSA infections are generally treated with intravenously administered vancomycin, although CA-MRSA isolates are typically resistant to fewer non- β -lactam antibiotics, and agents such as clindamycin, trimethoprim-sulfamethoxazole, and tetracyclines may be effective. Attempts at eliminating MRSA colonization of the body may also be helpful in preventing acquisition of infection in the hospital (e.g., at the time of surgery) or in preventing recurrent infection. Decolonization involves applying mupirocin to the nares (where MRSA is often carried) and washing with a skin antiseptic such as chlorhexidine or dilute bleach baths for 5 to 10 days. Hand hygiene, carefully cleaning surfaces that are frequently touched, avoiding sharing of personal items such as towels and razors, and keeping infected wounds covered are important for preventing the spread of MRSA. However, MRSA infections may recur despite preventive measures.

Paronychia

Paronychia is an infection of the cuticle surrounding the nail bed. Cases generally follow minor trauma, such as removing a hangnail. The involved part of the finger at the nail margin becomes painful, red, warm, and swollen, and pus may be expressed from around the nail bed. *Candida*, staphylococci, or streptococci are the usual causative organisms. Paronychia usually responds to warm soaks, which often lead to spontaneous drainage of pus and resolution. Systemic antimicrobial therapy and surgery may be needed in unusual cases.

Folliculitis

Folliculitis is an inflammation and infection of hair follicles. *S. aureus* is the most common causative agent of folliculitis, although *P. aeruginosa* has been implicated in cases acquired from contaminated swimming pools or hot tubs. In immunocompromised hosts, *Candida* spp., *Malassezia* spp., or gram-negative bacteria may cause folliculitis. Lesions appear as small, erythematous papules that may be pruritic, and they often evolve to form pustules with a whitish or yellowish central zone. Sycosis barbae is a form of folliculitis occurring in bearded areas of the face. The differential diagnosis of these folliculitis syndromes includes conditions that have a non-infectious etiology such as eosinophilic pustular folliculitis, seen in persons with acquired immunodeficiency syndrome (AIDS), or the folliculitis that is an adverse effect from certain targeted cancer immunotherapy agents.

Furuncles and carbuncles

Folliculitis can sometimes progress to form deeper inflammatory nodules called **furuncles** (Fig. 33.7), especially in warm, moist areas of the body and areas subjected to friction. Certain individuals are predisposed to the development of furuncles, including those with diabetes mellitus, obesity, or defects in immune function. Furuncles are initially red and firm but soon become painful and fluctuant and generally drain spontaneously. *S. aureus* is the most common causative pathogen. Furuncles usually can be managed by the application of moist heat to accelerate drainage, although systemic antimicrobial therapy may be needed depending on the severity.

A **carbuncle** is a more serious lesion that extends into the subcutaneous fat. It consists of multiple coalescing abscesses that can drain at several adjacent sites along hair follicles. Carbuncles commonly occur at the nape of the neck and on the back of the thighs and are often associated with fever and other systemic symptoms. Bacteremia can be a complicating event, and surgical drainage is needed for most carbuncles. Purulent drainage should be cultured to confirm the organism, which is typically *S. aureus*. As noted previously, because CA-MRSA has become a prevalent cause of skin and soft tissue infections, furuncles and carbuncles that are significant should be treated empirically with antimicrobial agents that have activity against MRSA. For milder infections, trimethoprim-sulfamethoxazole or tetracyclines can be used, and agents such as vancomycin, linezolid, or daptomycin are recommended for severe infections. If methicillin-susceptible *S. aureus* is cultured, dicloxacillin or cephalixin is effective.

S. aureus isolates exhibiting less susceptibility to vancomycin, known as *vancomycin intermediate-resistant S. aureus* (VISA), were discovered in the 1990s, and infections by VISA have been accompanied by failure of vancomycin therapy and poor outcomes. *S. aureus* isolates with complete resistance to vancomycin were first reported in the United States in 2002. However, resistance to vancomycin among *S. aureus* remains very rare in the United States despite its widespread use.

Hidradenitis suppurativa

Hidradenitis suppurativa is a difficult-to-treat, recurrent infection of the apocrine sweat glands. For unclear reasons, chronic obstruction of these glands, particularly in the axillae and groin, occurs in certain individuals, which predisposes



Fig. 33.7 *Staphylococcus aureus* furuncle of the breast.

to a secondary mixed bacterial infection of the skin and skin structures. Organisms such as staphylococci, streptococci, gram-negative bacteria (e.g., *E. coli* and *Pseudomonas*), and anaerobic bacteria are commonly involved. The disease manifests as nodular, tender, erythematous swellings that become fluctuant and drain, often associated with fever and tender lymphadenitis. Resolution of the lesions is typically accompanied by scarring of the involved areas. Individual episodes of infection are treated with local moist heat, broad-spectrum antimicrobial agents based on culture results, and frequently with surgical incision and drainage. Multiple recurrences of infection are often seen, which can lead to tissue fibrosis, sinus tract formation, and disfigurement.

Other soft tissue infections

Bite infections

Bites from humans or animals are often complicated by soft tissue infection that can be quite severe. Infections in this setting are generally polymicrobial, caused by a mixture of aerobic and anaerobic organisms found in the mouth of an animal or human inflicting the bite and/or human skin biota. Common pathogens implicated in human bite infections include *Streptococcus anginosus* group, *S. aureus*, *Eikenella corrodens*, *Fusobacterium nucleatum*, and *Prevotella melaninogenica*. Dog and cat bites account for about 1% of U.S. emergency department visits each year, with most caused by dog bites. The incidence of infection after a dog bite injury is between 2 and 25%. *Pasteurella*, *Bacteroides* spp., *Fusobacterium*, *Prevotella*, staphylococci, and *Capnocytophaga canimorsus* are common pathogens following dog bites. *C. canimorsus* may cause a severe sepsis syndrome in patients with liver disease or in those who are asplenic and can lead to **purpura fulminans**, similar to meningococcemia (see the “Immune-Mediated Cutaneous Disease” section later in this chapter).

Cat teeth can inflict very deep wounds that are difficult to irrigate and have a greater risk of infection, soft tissue abscess formation, and infection of underlying bones and joints compared with dog bites. It is estimated that 30% to 50% of cat bites result in infection, and cat bites progress to infection more rapidly than dog bites. Pathogens isolated after cat bites include *Pasteurella multocida*, *Bacteroides* spp., *Fusobacterium*,

Porphyromonas, and staphylococci. The cellulitis associated with bites can spread rapidly and leave permanent disability, particularly when the hand is involved.

Human bites are less common than animal bites and are most often seen in young children. Infections complicate 2% to 25% of human bites. For any bite wounds, wound care, including debridement, culture of deep tissue or purulent drainage, and prompt antimicrobial administration are important. Administration of antimicrobial agents at the time of the bite for 3 to 5 days is recommended to prevent infection in patients who have compromised immune systems or liver disease, or those with deep or severe injuries, particularly to the hand or face. Antimicrobial agents with aerobic and anaerobic activity are indicated for preemptive treatment or therapy of an established bite infection; amoxicillin-clavulanic acid, ampicillin-sulbactam, doxycycline, or a combination of a fluoroquinolone or trimethoprim-sulfamethoxazole with metronidazole are suitable regimens.

Diabetic foot infections

Foot infections are a very common occurrence in persons with diabetes mellitus; they account for a significant amount of morbidity. Risk factors for foot infection include the presence of peripheral neuropathy, trauma to the feet, inadequate blood glucose control, compromise of the peripheral vascular circulation caused by diabetes, impaired kidney function, and improper foot care, including walking barefoot. Manifestations that can occur include cellulitis, typically caused by streptococci or staphylococci (see the “Cellulitis” section earlier in this chapter); acute or chronic soft tissue ulceration, with or without underlying bone infection; and necrotizing infection or gangrene. Ulcerative lesions and gangrene may, like cellulitis, be the result of staphylococcal and streptococcal infection but often are mixed infections and include Enterobacteriales, *Pseudomonas*, gram-positive bacteria, and anaerobic bacteria. The impairment of host defenses seen in diabetic patients can also allow weakly virulent organisms, such as coagulase-negative staphylococci and diphtheroids, to be pathogens in the skin.

Cultures of open wounds are most helpful when they are obtained from deep tissues or from collections of pus beneath the skin surface. Superficial swabs yield little information because diabetic foot ulcers are often colonized with multiple bacteria, which may not necessarily be the agents causing infection. Cellulitis can be treated empirically with antimicrobial agents active against staphylococci and streptococci, whereas foot ulcers should generally be treated empirically with agents that also have gram-negative and anaerobic activity. Definitive antimicrobial therapy should be based on culture results from a deep specimen. Most diabetic foot wounds require debridement and may even result in amputation if blood flow to the area is poor and/or a non-response to wound care and antimicrobial therapy is seen. It is recommended that patients with diabetic foot infections have x-rays or magnetic resonance imaging of the affected area to assess whether there is infection of the bone, soft tissue gas (which signifies a necrotizing infection; see Fig. 33.8), or foreign bodies, and the affected leg should be assessed for adequacy of blood flow.

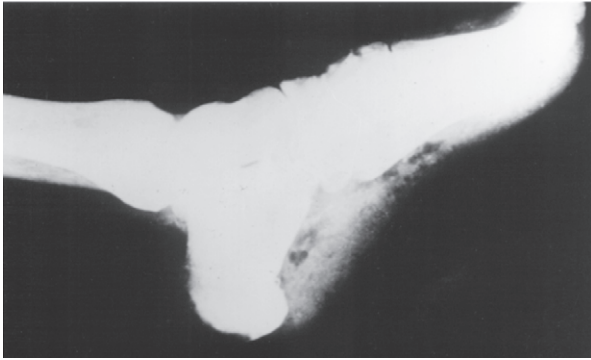


Fig. 33.8 Diabetic foot infection with soft tissue gas formation.

Case check 33.1

Patients with diabetes mellitus are at high risk for skin and soft tissue infections. Elevated blood glucose levels can impair the body's immune function and make it more difficult to recover from infections. The diabetic patient in the Case in Point developed a polymicrobial infection of the subcutaneous tissue that gave rise to gram-negative sepsis.

Necrotizing soft tissue infection

Infections that produce tissue necrosis and soft tissue gas represent an important subset of skin and soft tissue infection cases. Rapidly progressing and frequently life-threatening, these soft tissue infections can be classified according to their level of soft tissue involvement (e.g., superficial, epidermal, or dermal structures; fascia; muscle) and according to the causative microbiologic agent or agents. Subtypes of necrotizing infection (infectious gangrene) include polymicrobial **necrotizing fasciitis**, typically caused by Enterobacteriales and anaerobes (type I); monomicrobial necrotizing fasciitis, usually caused by *S. pyogenes*; or less commonly, *S. aureus* (type II) and **gas gangrene** (type III, also known as *clostridial myonecrosis*) caused by *Clostridium* spp., usually *Clostridium perfringens* (Fig. 33.9). *Vibrio vulnificus* and *Aeromonas hydrophila* are additional causes of necrotizing soft tissue infection. *Vibrio* infection can occur through contamination of a traumatic injury with seawater or through consumption of raw oysters, and *Aeromonas* infection can occur via medicinal leech therapy or through freshwater contamination of an injury.

Necrotizing soft tissue infections often begin at a site of trauma but may also occur without any obvious portal of entry or as the result of a bacteremia with secondary involvement of the skin and soft tissues. The most common form of necrotizing soft tissue infection is type I, which tends to occur in the trunk and perineal region of patients who have underlying medical illnesses such as diabetes, are immunocompromised, or have had surgery. Genital or perineal necrotizing infection is known as *Fournier's gangrene*. Patients with type II infection can be younger and previously healthy, and infections more often involve the extremities. Blunt trauma, surgery, childbirth, and IV drug use are risk factors for type II infection. Risk factors for necrotizing infection caused by MRSA include IV drug use, diabetes, human immunodeficiency virus (HIV) infection, and malignancy.



Fig. 33.9 Clostridial myonecrosis (gas gangrene). (From Finegold, S. M., et al. [1986]. Anaerobic infections. Kalamazoo, MI: Upjohn.)

With certain organisms (e.g., *S. pyogenes*, *S. aureus*, or *Clostridium* spp.), the pathophysiology of necrotizing infection involves toxin production and induction of inflammatory cytokines leading not only to extensive soft tissue damage but also systemic effects like shock and multiorgan failure. Toxic shock syndrome may occur in the setting of necrotizing infection caused by *S. pyogenes* or *S. aureus* (see the "Toxic Shock Syndrome" section later in this chapter).

Initially, pain at the involved site is out of proportion to the physical findings. Hemorrhage and necrosis of the skin ensue, with formation of bullae and palpable gas (crepitus) caused by tissue ischemia from thrombosed blood vessels. Loss of sensation of the involved area of skin and soft tissue may occur. Certain laboratory findings can support the diagnosis of necrotizing fasciitis; the white blood cell count is often more than $15 \times 10^9/L$, and there may be increased levels of C-reactive protein, lactate, creatinine kinase, creatinine, and glucose and decreased levels of serum sodium or hemoglobin. Necrotizing soft tissue infection often progresses from superficial to deep tissue involvement in a rapid manner, risking limb loss, systemic toxicity, and death.

Gangrenous soft tissue infections represent true medical and surgical emergencies. Immediate surgical exploration is required to determine the degree and level of soft tissue spread, excise all devitalized tissue, and obtain deep tissue specimens for Gram stain and bacterial cultures. Blood cultures may also be positive. Early surgical debridement is the major determinant of patient survival. However, before surgical exploration and debridement, combination antimicrobial therapy should be initiated and should include vancomycin or daptomycin plus piperacillin-tazobactam or a carbapenem.

Case check 33.2

Skin infections in diabetic persons may evolve into the rapidly progressive type I necrotizing fasciitis, in which infection extends through the subcutaneous tissues to the fascia. Necrotizing soft tissue infections are associated with fever, leukocytosis, and shock, requiring emergent surgical debridement and antimicrobial therapy. Infections of this type are polymicrobial, caused by aerobic gram-positive organisms, including staphylococci and streptococci, as well as Enterobacteriales and anaerobic bacteria.

plus clindamycin, which can inhibit toxin production by streptococci and staphylococci. Empiric therapy should be active against aerobic gram-positive cocci including MRSA, Enterobacterales, and anaerobes to ensure adequate coverage.

Mycetoma

Mycetoma (also called *Madura foot* or *maduromycosis*) is a chronic skin and subcutaneous infection caused by either bacteria or fungi. It is known as *eumycetoma* or *true fungal infection* when caused by fungi, such as *Madurella* spp., *Aspergillus* spp., or *Pseudallescheria boydii*, and as *actinomycetoma* when caused by filamentous bacteria, including actinomycetes such as *Nocardia* or *Actinomadurea* spp. (Table 33.4).

Madura foot is characterized by localized swelling and abscess formation within subcutaneous tissues and the development of sinus tracts, with visible grains/granules that are 0.2 to 0.5 mm in size formed by aggregates of organisms in the pus draining from these fistulae (Fig. 33.10). The disease progresses slowly and is destructive, with extension to muscle and bone. Soil, plants, and decaying vegetation are reservoirs for the causative organisms. The disease is often seen in outdoor laborers and may be a result of lack of protective clothing, including shoes; it develops as a result of skin trauma with an object contaminated with fungal elements (e.g., thorns, splinters) or introduction of organisms into preexisting abrasions. There is no person-to-person transmission.

Table 33.4 Major causative agents of subcutaneous mycoses

Disease	Principal organism(s)
Eumycetoma	<i>Madurella</i> spp., <i>Blastosporium</i> spp., <i>Scedosporium apiospermum</i> complex, <i>Leptosphaeria senegalensis</i> , <i>Exophiala</i> spp., <i>Curvularia</i> spp., <i>Phialophora verrucosa</i> , <i>Pyrenochaeta romeroi</i> , <i>Fusarium</i> spp., <i>Acremonium</i> spp., <i>Pseudallescheria boydii</i> , <i>Aspergillus</i> spp., <i>Neotestudina rosatii</i> , <i>Pleurostomophora ochracea</i> , <i>Trematosphaeria grisea</i>
Actinomycetoma	<i>Actinomadurea madurae</i> , <i>Actinomadurea pelletieri</i> , <i>Nocardia</i> spp., <i>Streptomyces somaliensis</i>
Chromoblastomycosis	<i>Phialophora verrucosa</i> , <i>Fonsecaea pedrosoi</i> , <i>Fonsecaea compacta</i> , <i>Fonsecaea monophora</i> , <i>Cladophialophora carrionii</i> , <i>Rhinocladiella aquaspersa</i> , <i>Exophiala</i> spp.
Phaeohyphomycosis	Dematiaceous fungi (e.g., <i>Phialophora</i> , <i>Wangiella</i> , <i>Exophiala</i> , <i>Exserohilum</i> , <i>Alternaria</i> , <i>Cladosporium</i> , <i>Curvularia</i> , <i>Bipolaris</i>)
Sporotrichosis	<i>Sporothrix schenckii</i>
Rhinoentomophthoromycosis	<i>Conidiobolus</i> , <i>Basidiobolus</i>
Lobo disease	<i>Lacazia loboi</i> (<i>Loboia loboi</i>)

The foot is the most commonly affected site (70%) followed by the lower leg and hand. Other sites of infection can include the shoulder and back, with the face rarely involved. The infected sites are usually nontender, and no systemic signs of infection occur. Mycetoma is rare in the continental United States but common in tropical and subtropical regions of the world, especially where people walk barefoot. It is unclear whether there are specific genetic predispositions to mycetoma, but it is not seen in classically immunocompromised persons.

The diagnosis of mycetoma is usually made based on the clinical appearance and microscopic examination and culture of the grains. Aspiration of an area of soft tissue swelling for microscopy and culture can also be performed. Treatment with antimicrobials should be prolonged, and surgery to debulk the disease is often necessary. Amputation may be required for cure. Eumycetomas respond best to azole antifungal agents such as itraconazole, voriconazole, posaconazole, or isavuconazole. Actinomycetoma may be treated with trimethoprim-sulfamethoxazole and may sometimes require combination antimicrobial therapy with amikacin. Other agents that may have activity in the treatment of actinomycetoma include imipenem, minocycline, amoxicillin-clavulanate, linezolid, and the fluoroquinolones.

Mycetoma may be difficult to distinguish from chronic osteomyelitis and botryomycosis, the latter being a clinically and pathologically similar entity caused by a variety of bacteria, including staphylococci and gram-negative bacteria. Specific diagnosis depends on visualizing the granules in fresh preparations or histopathologic sections and isolating the causative actinomycete or fungus in culture. Different causative agents produce differently colored and sized granules, which can help in determining the etiologic organism.

Chromoblastomycosis

Chromoblastomycosis or chromomycosis is a chronic spreading mycosis of the skin and subcutaneous tissues. It produces localized wartlike, scaly lesions, usually of a lower extremity (Fig. 33.11) or, less commonly, the hand or back. The disease has been found worldwide but, like mycetoma, is most common in tropical and subtropical regions. The mode of transmission is also like that of mycetoma and occurs through skin inoculation of organisms via minor trauma. Progression is



Fig. 33.10 Madura foot. (Courtesy Dr. Gail Reid, Loyola University Medical Center, Maywood, IL.)



Fig. 33.11 Chromoblastomycosis of the leg. (Courtesy Dr. Gail Reid, Loyola University Medical Center, Maywood, IL.)

slow over a period of years, with eventual large cauliflower-like masses and obstruction of lymphatic drainage, in some cases leading to marked swelling of the affected site. Unlike mycetoma, chromoblastomycosis generally does not involve muscle or bone.

Infectious agents include the dark-walled (dematiaceous) fungi found in soil and decaying vegetation. *Fonsecaea pedrosoi* is usually isolated, although other potential pathogens include *F. monophora*, *F. compacta*, *Phialophora verrucosa*, *Cladophialophora carrionii*, and *Rhinocladiella aquaspersa*. Rare cases caused by *Exophiala* spp. have also been described, and the climate of a region may predispose to infection by a specific chromoblastomycosis agent. Microscopic examination of scrapings or biopsy specimens from lesions reveals characteristic brown, thick-walled, rounded cells known as *sclerotic* or *copper penny bodies* that divide by fusion in two planes. Confirmation of the diagnosis is made by biopsy and culture of the causative fungus on SDA, typically resulting in dark, pigmented colonies of fungus that may take weeks to grow. Excision of localized lesions provides optimal results. For more extensive skin involvement, a combined approach of antifungal agents such as itraconazole or terbinafine along with debridement where feasible and/or application of liquid nitrogen or heat may be effective. Voriconazole, posaconazole, and isavuconazole display in vitro activity against many of the agents of chromoblastomycosis.

Other uncommon fungi

The term **phaeohyphomycosis** refers to infections caused by fungi that produce dark cell walls. These fungi are also known as *dematiaceous*, again referring to their brown or black pigmentation. In addition to causing some cases of mycetoma and chromoblastomycosis, the agents of phaeohyphomycosis can cause subcutaneous nodules (as well as deeper infections such as brain abscess, septic arthritis, pneumonia, and sinusitis). *Curvularia* and the closely related genus *Bipolaris*, *Exophiala*, and *Phialophora* are some of the common genera isolated from skin lesions, although many others constitute this group. They are found in soil and decaying organic matter and on plants and are usually introduced into the body in a manner like mycetoma and chromoblastomycosis, through trauma and breaks in the skin.

Subcutaneous phaeohyphomycosis starts as an erythematous nodule, often on the extremities, at a site of minor trauma. The lesion typically enlarges and may involve deep tissues, including bone. The infection can spread locally with the formation of additional nodules. Immunocompromised persons are at highest risk for this infection, and in this setting the infection can disseminate to other regions of the body, including the brain. The diagnosis is made on biopsy and culture of a skin lesion, although the dark pigment of the organisms may not be easily visualized on hematoxylin and eosin tissue staining and may require staining by the Fontana-Masson method. Surgical debridement is generally necessary for cure; several antifungal agents are active against these organisms, including amphotericin B (usually reserved for life-threatening cases), itraconazole, voriconazole, and posaconazole.

Mucormycosis is an infection caused by fungi that belong to the order Mucorales. Mucorales organisms are ubiquitous in nature and have broad, irregular hyaline hyphae with rare septations. They may be found in food, vegetation, animal excreta, and soil. The disease resulting from these agents is termed *mucormycosis*. Within the order Mucorales, disease is caused most often by the genus *Rhizopus*, although *Mucor*, *Rhizomucor*, *Cunninghamella*, *Apophysomyces*, and *Lichtheimia* (formerly *Absidia* and *Myocladus*) are other common genera identified in human infections. Disease results less frequently from genera such as *Saksenaia* and *Syncephalastrum*.

Patients typically develop localized cutaneous mucormycosis via skin inoculation from trauma, including from burns, surgery, or IV drug use. Contaminated bandages and bed linens have also resulted in nosocomial outbreaks of skin and soft tissue mucormycosis. The more common mode of acquiring the infection is through inhalation of fungal spores from the environment. Ingestion is another route; the skin can then also be involved by dissemination from the lungs, sinuses, brain, or gastrointestinal (GI) tract (see the “Dermatologic Manifestations of Systemic Bacterial and Fungal Infections” section later in this chapter). Risk factors for infection include uncontrolled diabetes mellitus, leukemia, bone marrow and solid organ transplantation, corticosteroid therapy, and exposure to the iron chelator deferoxamine, which can make iron more available for use by the fungus. Disseminated mucormycosis has a high fatality rate.

Cutaneous lesions are often erythematous and develop black, necrotic areas. They can progress quickly. The diagnosis is made by biopsy of tissue for histologic examination and culture. The finding of irregular, aseptate hyphae branching at 90-degree angles suggestive of mucormycosis in the tissue of a diabetic or immunocompromised patient is a critical value, and the patient’s health care provider should be notified immediately. Mucorales often grow quickly on SDA, and morphologic features of fungal growth can allow genus identification. Matrix-assisted laser desorption/ionization–time-of-flight (MALDI-TOF) mass spectrometry and polymerase chain reaction (PCR) are increasingly used for identification when fungi cannot be identified morphologically. The mainstay of treatment for mucormycosis is surgical debridement in combination with amphotericin B, although susceptibility to amphotericin B varies by species. Posaconazole and the newest triazole, isavuconazole, also have activity against Mucorales.

Fluconazole and voriconazole are not clinically useful, and echinocandins alone are not active against Mucorales.

Fungi of the order Entomophthorales are also present in organic debris and can cause **entomophthoromycosis**, a chronic subcutaneous form of infection. This is often the result of traumatic implantation of the organisms in the skin. Entomophthoromycosis is caused by organisms of two genera: *Conidiobolus* (*C. coronatus*, *C. incongruus*) and *Basidiobolus* (*B. ranarum*). Entomophthoromycosis is most often seen in tropical and subtropical regions, especially in Africa, Southeast Asia, and Latin America. *B. ranarum* causes subcutaneous infection of the limbs or trunk, particularly the shoulders, hips, thighs, and buttocks. It is characterized by nontender rubbery masses that can be large and ulcerate and is predominantly a disease of early childhood. *Conidiobolus* infection is localized to the facial area, typically in adults. *Conidiobolus* infections occur after inhalation of fungal spores that subsequently invade the sinuses and facial soft tissues and cause painless swelling of the lips and face that can be very deforming. Occasionally, these infections may spread to other areas of the body, particularly in immunocompromised persons.

Diagnosis can be made clinically based on the typical skin and soft tissue findings but should be confirmed with biopsy, which shows broad hyphae with few septations and surrounding eosinophils. Culture of tissue obtained from biopsy should also be performed but may be negative. *Conidiobolus* is less susceptible to antifungal therapy than *Basidiobolus* and may require debridement along with a combination of itraconazole and potassium iodide. Itraconazole is active against *Basidiobolus*. Limited data are available on the utility of newer triazoles, but posaconazole and voriconazole may have activity.

Lobomycosis is a chronic fungal infection of the skin caused by *Lacazia loboi* (previously known as *Loboa loboi*). This infection is mainly found in the tropics of South and Central America, with sporadic cases in Africa and other areas of the world. The organisms are thought to reside in soil or vegetation and infect humans via skin trauma. Farmers, hunters, and jungle workers are affected more than others. Dolphins can also be infected, so it is presumed that *L. loboi* also exists in bodies of water. The disease occurs as slowly forming skin nodules of various sizes that occur on the face, arms, legs, and feet. The lesions can differ in appearance and can be flat or nodular and warty or ulcerated. Infection can exist over a period of years, with slow progression of the disease, which is relatively asymptomatic. Special staining of lesion biopsy material is required to demonstrate the characteristic short chains of lemon-shaped fungal cells. *L. loboi* has never been isolated in culture. The only effective treatment is surgical excision, although clofazimine may be partially effective. The infection is generally resistant to therapy with the antifungal medications typically used to treat other invasive cutaneous mycoses, although posaconazole has proven successful in at least one case.

Nodular lymphangitis

Nodular lymphangitis, or lymphocutaneous syndrome, is an entity characterized by inflammatory nodules that occur along lymphatic vessels that drain an area of primary skin infection (Fig. 33.12). Certain organisms are commonly associated with nodular lymphangitis, including *Sporothrix*

schenckii, *Nocardia*, and mycobacteria, although other, more unusual infectious agents can also be involved (Box 33.2).

Sporotrichosis

Sporotrichosis is the most frequently recognized cause of nodular lymphangitis. The agents of this disease include *S. schenckii* as well as the more newly identified species *S. brasiliensis*, *S. globosa*, and *S. luriei*, each of which has unique epidemiologic and virulence characteristics. *S. brasiliensis* was initially confined to Brazil, although it has since been found in other areas of South



Fig. 33.12 Nodular lymphangitis caused by sporotrichosis with ulceration of the overlying skin. (Courtesy Dr. Gail Reid, Loyola University Medical Center, Maywood, IL.)

BOX 33.2 Microbiological causes of lymphocutaneous syndromes

Fungi

Sporothrix schenckii
Blastomyces dermatitidis
Histoplasma capsulatum
Coccidioides immitis
Scopulariopsis spp.
 Actinomycetes
Nocardia spp.

Parasites

Leishmania spp.

Mycobacteria

Mycobacterium marinum
Mycobacterium kansasii
Mycobacterium chelonae
Mycobacterium abscessus
Mycobacterium fortuitum

Bacteria

Staphylococcus aureus
Francisella tularensis
Bacillus anthracis

Viruses

Herpes simplex virus

America. *S. globosa* is uncommon, but it has been identified throughout the world and is the main cause of sporotrichosis in China. For the purposes of this discussion, we will use the term *S. schenckii* complex to refer to all species of *Sporothrix*. *Sporothrix* complex organisms are known as *dimorphic* because of their ability to exist either as a yeast (at higher temperatures, as in human tissue) or in hyphal form (in the environment, at temperatures <37° C). Sporotrichosis is typically an occupational disease of gardeners, farmers, and horticulturalists. Introduction of the fungus through the skin occurs by pricks of thorns or barbs; handling sphagnum moss or slivers from wood or other material contaminated with the organism has also been identified as a risk for infection. However, *S. brasiliensis* and, to a lesser extent, *S. schenckii* display zoonotic transmission, with cats being the source.

Sporotrichosis is usually localized to the skin and subcutaneous tissues. However, dissemination to bone, lungs, brain, and other organs can occur in immunocompromised persons, and pulmonary disease can occur when *S. schenckii* complex conidia are inhaled. Cutaneous disease begins as an erythematous painless nodule at the inoculation site, which may ulcerate and remain confined (cutaneous form) or grow and spread proximally, with surrounding satellite nodules along the lymphatic channels (lymphocutaneous form or nodular lymphangitis). Laboratory confirmation of sporotrichosis is made by culture of pus or exudate on SDA, preferably aspirated from an unopened lesion, and growth may take up to 30 days. The characteristic oval- to cigar-shaped yeasts are rarely visualized by direct smear of pus because the organisms are often few, but Grocott-Gomori methenamine silver and other fungal stains of tissue obtained from biopsy are useful in identifying the fungus. Molecular tools such as PCR may be required to distinguish the species.

Itraconazole is the first-line treatment for cutaneous sporotrichosis. Orally administered terbinafine also has activity and is an alternative therapy. There is less experience with newer azoles, but posaconazole has good in vitro activity, unlike isavuconazole. More severe or disseminated disease is treated with amphotericin B. Heating tissues to 42° to 43°C has also been effective in decreasing the size of lesions because *S. schenckii* complex grows best at lower temperatures. Local hyperthermia may be useful for mild cutaneous disease in pregnant women to avoid the toxicity of antifungal therapy.

Nocardiosis

Nocardia spp. are ubiquitous in the environment and may be found in soil, water, and vegetation. They are part of the aerobic actinomycetes group that also includes *Corynebacterium*, *Rhodococcus*, *Gordonia*, *Tsukamurella*, *Streptomyces*, *Actinomadura*, and *Mycobacterium*. More than 40 characterized *Nocardia* spp. can cause human disease; in recent years, molecular techniques continue to identify new species causing infection in humans. Some of the most common recently described species reported include *N. veterana*, *N. abscessus*, *N. paucivorans*, and *N. cyriacigeorgica*. There is geographic variation in the frequency of different species.

Nocardia spp. can cause localized or disseminated infection, with the latter occurring more often in immunocompromised hosts. In the skin and soft tissue form of nocardiosis

that results from direct cutaneous inoculation, manifestations range from lymphocutaneous syndrome to subcutaneous abscesses, cellulitis, and mycetoma. The skin can also be involved through hematogenous dissemination from another source. Although *N. cyriacigeorgica*, *N. nova*, and *N. farcinica* cause most noncutaneous invasive disease, *N. brasiliensis* is the most common species causing cutaneous or lymphocutaneous nocardiosis, particularly in tropical countries. A variety of other species can also cause cutaneous disease.

Direct smears from nocardial skin lesions will show gram-positive, beaded, branching filaments. The organisms are acid-fast stain negative but can be seen with a modified acid-fast stain that uses a weaker acid to decolorize the primary stain carbol fuchsin. This feature, as well as its aerobic growth, can distinguish *Nocardia* from organisms such as *Actinomyces* that is both acid-fast and modified acid-fast negative and grows anaerobically. *Nocardia* spp. may take as long as 2 weeks to grow in blood culture media. The treatment of choice is trimethoprim-sulfamethoxazole, although other antimicrobials, such as imipenem, linezolid, and amikacin, also have good activity. There are important species differences in antimicrobial susceptibilities, with some species such as *Nocardia farcinica* being relatively resistant to several antimicrobials, so it is important to perform susceptibility testing of clinical isolates.

Mycobacterial infection

Nontuberculous mycobacteria

Nontuberculous mycobacteria (NTM) are distributed throughout the environment, and many species are known to cause cutaneous disease, including nodular lymphangitis. Skin and soft tissue infection is the most common extrapulmonary manifestation of disease caused by NTM, and recent data indicate an increasing incidence of NTM cutaneous infection over the past several decades. Puncture wounds, motor vehicle accidents, injections, cosmetic procedures including tattooing, and other surgeries are the typical precipitating factors for skin infection, although hematogenous spread to the skin from other sites can occur (see later).

Mycobacterium marinum, a marine organism; *M. ulcerans*; and rapidly growing mycobacteria (RGM) including *M. abscessus* complex, *M. chelonae*, and *M. fortuitum* group are the most common NTM isolated from skin lesions. *M. marinum* skin infection, also known as *swimming pool* or *fish tank granuloma*, develops in individuals with a history of cleaning fish tanks or exposure to saltwater or nonchlorinated swimming pools. The microorganism enters through an open wound or via traumatic inoculation of the skin. The lesions are initially solitary, red-purple in color, and located on the hands or arms 2 to 3 weeks after exposure. They are papular or nodular and can become wartlike or ulcerated. The infection can then progress to a lymphocutaneous syndrome characterized by the development of more proximal lesions along the path of lymphatic drainage of the initial site of infection.

The diagnosis is made by biopsy and culture of a skin lesion in association with a history suggestive of exposure. *M. marinum* grows best at 25° to 32° C. Treatment involves systemic antimycobacterial therapy for several months, until resolution of the lesions occurs. Combination antimicrobial

therapy with clarithromycin plus ethambutol or rifampin is generally used, although in more superficial skin infections, clarithromycin or doxycycline monotherapy may be successful. *M. marinum* is also susceptible to trimethoprim-sulfamethoxazole and linezolid. Applying local heat has also been used as an adjunctive therapy.

Lesions caused by RGM can appear as nodules, papules, plaques, or ulcers. *M. chelonae* is usually seen in immunocompromised patients, whereas cutaneous infections caused by *M. fortuitum* complex typically occur in otherwise healthy persons. *M. abscessus* complex infections are seen in both patient types. Exposure to acupuncture, dermal filler injections, tattooing, nail salons, and mesotherapy have been risk factors for infection. Healthcare-associated skin infections from steroid injections or contaminated antiseptics have occurred.

M. ulcerans infection usually occurs as a single pruritic ulcer with undermined edges, also known as a *Buruli ulcer*. *M. ulcerans* infection is not endemic in the United States but is associated with swamps and may cause a chronic infection with limb deformity in tropical climates. It grows very slowly in culture and can take up to 12 weeks of incubation. Early disease can be cured with surgical debridement.

Diagnosis of cutaneous NTM infections is made by acid-fast staining and culture of skin biopsy or drainage material. Molecular methods for species identification including partial gene sequencing or the use of gene probes is often necessary, and MALDI-TOF mass spectrometry may also be useful in determining species. Antituberculous antimicrobials do not have significant activity against most of these NTM, but antimicrobials such as fluoroquinolones, clarithromycin, aminoglycosides, imipenem, doxycycline, and cefoxitin may be used, depending on the species.

Mycobacterium tuberculosis

Cutaneous tuberculosis is uncommon, but inoculation of the skin with *M. tuberculosis* can lead to tuberculosis verrucosa cutis (TVC). TVC lesions develop as red or purple papules that become wartlike. They occur in areas of the body prone to trauma. Historically, manifestation of TVC by *M. tuberculosis* was seen in physicians and anatomists who infected themselves by contact with patients or cadavers (*prosector's wart*). In the tropics, TVC is seen in children who walk barefoot in soil contaminated with tuberculous sputum. Tuberculous chancre, a skin ulcer, is another localized dermatologic manifestation of *M. tuberculosis* following traumatic inoculation of the skin with the organisms. Cutaneous tuberculosis can also result from suppuration of an infected lymph node or bone/joint with extension to the skin, known as *scrofuloderma*. Treatment of cutaneous tuberculosis is with standard antituberculous antibiotics.

Actinomycosis

Actinomycosis is a chronic disease characterized by the formation of abscesses, tissue fibrosis, and draining sinuses that discharge sulfur granules (yellow-colored masses of organisms). It is caused by non-spore-forming anaerobic or microaerophilic bacterial species, especially *Actinomyces israelii*, although a variety of *Actinomyces* species have been identified as causing human infection. Once thought to be



Fig. 33.13 Actinomycosis of the jaw following dental infection. (Courtesy Dr. Gail Reid, Loyola University Medical Center, Maywood, IL.)

fungi because of their branching, *Actinomyces* spp. and the closely related *Nocardia* spp. are classified as higher prokaryotic bacteria. *Actinomyces* spp. are gram-positive, pleomorphic, and diphtheroidal or, more commonly, delicately filamentous. They are part of the normal biota of the mouth and GI and genital tracts. Although there are thoracic, abdominopelvic, and central nervous system (CNS) forms of the disease, involvement of the face and neck is the most common manifestation (Fig. 33.13) and often follows dental sepsis or manipulation, trauma, tonsillitis, otitis, or mastoiditis. Cervicofacial actinomycosis may extend to the underlying mandible or facial bones, leading to osteomyelitis. Penicillin is the preferred treatment.

Dermatologic manifestations of systemic bacterial and fungal infections

Systemic infections can produce skin lesions that may provide important diagnostic clues. This phenomenon may be seen in various bacterial, fungal, parasitic, and viral infections. In this section, some important dermatologic findings associated with systemic bacterial and fungal infections are discussed. Skin lesions associated with viral and parasitic infections are described later in this chapter.

Bacteria

Pseudomonas infection

Ecthyma gangrenosum is a characteristic skin lesion associated with *Pseudomonas* bacteremia. The lesions begin as painless, flat, erythematous areas, often on the legs, that progress rapidly to nodules and then bullae that subsequently ulcerate and form a black eschar on the surface, with surrounding erythema. Although ecthyma gangrenosum has classically been associated with *Pseudomonas* sepsis, such lesions have also been reported in sepsis caused by other gram-negative bacteria, *S. aureus* and *Candida*. Ecthyma gangrenosum is the result of bacterial invasion of dermal blood vessels leading to ischemia, hemorrhage, and necrosis. Biopsy and Gram-stained

smear of the lesions reveals bacteria in the tissue, and inflammation of blood vessels is also present.

Vibrio and Aeromonas infections

Vibrio vulnificus is a gram-negative organism that is part of the ocean microbiota and can cause severe disease with a high fatality rate, particularly in persons with underlying liver dysfunction. Persons with other chronic diseases such as diabetes mellitus, hemochromatosis, renal failure, or alcohol abuse are also at increased risk. Infection occurs through ingestion of contaminated shellfish, such as raw oysters, or through exposure of an existing wound to seawater containing the organisms. Patients with *V. vulnificus* sepsis can develop widespread skin lesions characterized by the formation of hemorrhagic bullae that evolve into ulcers with skin necrosis.

V. vulnificus grows well on MacConkey agar but can be overlooked among other gram-negative organisms because it usually is a lactose fermenter. In addition, *Vibrio* spp. can be isolated from stool specimens, but isolation and identification are not routinely performed in many clinical laboratories and must be specifically requested. Commercially available stool PCR panels such as the BioFire FilmArray Gastrointestinal Panel (BioFire Diagnostics) can identify *Vibrio* spp.

Aeromonas spp. are found in freshwater and brackish water and can cause skin lesions similar to those caused by *V. vulnificus*. However, skin infection caused by *Aeromonas* is often the result of contamination of a preexisting wound with water containing the organisms. Aeromonads cause a variety of infections in addition to cellulitis, including gastroenteritis, peritonitis, and cholangitis. These infections may be complicated by bacteremia and sepsis, and patients with underlying liver disease are at higher risk for invasive *Aeromonas* infection. *Aeromonas* skin infection is typically rapidly progressing, with formation of hemorrhagic bullae and skin necrosis. Osteomyelitis and myonecrosis with gas formation in the tissues may ensue, and debridement is required in this setting. *Aeromonas* infection is also a well-recognized complication of medicinal leech therapy used in reimplantation or flap procedures because aeromonads are symbionts within the leech gut. Prophylactic antimicrobial agents, typically fluoroquinolones, trimethoprim-sulfamethoxazole, or third-generation cephalosporins are advised for patients receiving leech therapy. *Aeromonas* spp. can be cultured on MacConkey agar and are oxidase-positive, which distinguishes these organisms from most Enterobacterales.

Borreliosis

The predominant tickborne spirochete of Lyme borreliosis (disease), *Borrelia burgdorferi*, characteristically produces a distinctive “bull’s eye” or target skin lesion called *erythema migrans* (EM) at the inoculation site 1 to 2 weeks after infection (Fig. 33.14). The lesions are at least 5 cm in size and may become hemorrhagic or necrotic in the center. EM is the most useful diagnostic marker where it occurs in 70% to 80% of persons with Lyme borreliosis. Over a period of weeks, multiple secondary EM lesions can occur in various regions of the body as the spirochetes disseminate. Other manifestations of Lyme borreliosis include arthritis, carditis, and neurologic

disease. In addition to recognizing EM, the diagnosis of Lyme borreliosis can be made by serologic testing to identify antibodies against *B. burgdorferi*. Ceftriaxone, penicillin G, amoxicillin, and tetracycline antibiotics are useful in the treatment of Lyme borreliosis.

A rash very similar to EM can occur in southern tick-associated rash illness (STARI). This is a disease of unknown cause that can occur after a tick bite from the lone star tick, *Amblyomma americanum*. The *Ixodes scapularis* tick, not the lone star tick, is responsible for most of the *B. burgdorferi* transmission in the United States, but *I. pacificus* ticks can also transmit *B. burgdorferi* in the Pacific Northwest. Lyme borreliosis is endemic in the upper Midwest, Northeastern, and mid-Atlantic United States. Lone star ticks are found in the eastern, southeastern, and south-central areas of the United States.

A nonspecific petechial, macular, or papular rash is commonly seen during the end of the primary febrile episode of relapsing fever caused by *Borrelia recurrentis*. The disease is transmitted by human body lice and affects predominantly homeless persons or those in conditions of poor hygiene. It has become uncommon in the United States and is now primarily found in northeastern Africa. Diagnosis of relapsing fever is made by demonstrating *Borrelia* organisms in the peripheral blood of febrile patients using dark-field microscopy or Giemsa- or Wright-stained thick and thin blood smears.

Treponema infection

Syphilis is a sexually transmitted infection caused by *Treponema pallidum* subsp. *pallidum* that may be acute or chronic. Characteristic clinical manifestations differ depending on the length of time that has elapsed since infection. Cases of syphilis have been on the increase in the United States, particularly among men who have sex with men, especially those with HIV infection. The primary lesion usually appears as a papule at the inoculation site 1 to 3 weeks after the initial exposure, though it can occur as late as 3 months after exposure. Erosion and ulceration then occur, forming the characteristic painless



Fig. 33.14 Erythema migrans rash of Lyme borreliosis (disease). (Courtesy Dr. Gail Reid, Loyola University Medical Center, Maywood, IL.)

syphilitic chancre (Fig. 33.15) that is indurated or rubbery, in contrast with the painful soft chancre of chancroid caused by *Haemophilus ducreyi*. Lesions often occur on the genitalia but can develop at any site of inoculation, including the mouth, rectum, or hands. Multiple chancres can occur, particularly in those with HIV infection.

Spontaneous resolution of the primary lesion(s) occurs within several weeks, and this is followed by the secondary stage of syphilis 2 to 8 weeks later. Secondary syphilis usually involves the skin and mucous membranes, although inflammation of organs such as the liver, CNS, bones, or eyes may occur. The rash of secondary syphilis is characterized as a diffuse, painless, maculopapular rash (Fig. 33.16), and involvement of the palms and soles is characteristic. Highly infectious lesions known as *mucous patches* may occur on the lips, oral mucosa, or genital mucosa. Systemic complaints, including fever, malaise, and enlarged lymph nodes, commonly accompany secondary syphilis.

Secondary manifestations also disappear spontaneously within weeks. Subsequently, the infection remains clinically latent for weeks to years and may evolve into tertiary disease affecting predominantly the CNS and heart. Diagnosis can be



Fig. 33.15 Penile syphilitic chancre caused by *Treponema pallidum* subsp. *pallidum*.



Fig. 33.16 The rash of secondary syphilis.

made based on physical findings and serologic tests. *T. pallidum* cannot be detected by Gram stain but can be visualized by darkfield microscopy, a technique that is not widely available in laboratories. Sometimes, spirochetes can be seen on silver-stained biopsy materials, but silver staining of biopsy materials is rarely performed. *T. pallidum* cannot be grown on artificial media. Serologic methods are often used to diagnose infection.

Zoonoses

Zoonotic diseases are those transmitted to humans by wild or domestic animals. There are hundreds of zoonotic diseases. This section examines some of the more common systemic bacterial **zoonoses** that have significant dermatologic manifestations.

Rickettsiosis

Dermatologically, rickettsial infections are characterized by the type and distribution of the associated rash (e.g., petechial or vesicular; centripetal; or centrifugal) and the presence or absence of a black eschar at the vector bite inoculation site. Clinical symptoms common to rickettsial infections include high-grade fever, chills, malaise, headache, myalgias, rash, and conjunctival injection. Systemic and cutaneous disease manifestations are the result of widespread inflammation of small blood vessels (**vasculitis**).

Rickettsiae are gram-negative intracellular pathogens that reside within endothelial cells and macrophages. Rickettsiae have various animal reservoirs and are transmitted to humans through several species-specific insect vectors (e.g., ticks, mites, lice, fleas). There are two general groups of rickettsiae: the spotted fever group, which are predominantly transmitted by ticks with a few exceptions, and the typhus group, transmitted by insects (fleas or lice). There are more than 30 species of rickettsiae including two typhus group species, and many are human pathogens.

Rocky Mountain spotted fever (RMSF), caused by *Rickettsia rickettsii*, is the most frequently seen rickettsial infection in the United States and is transmitted by several different species of ticks in the genera *Dermacentor*, *Amblyomma*, and *Rhipicephalus*, depending on geographic location. Contrary to its name, RMSF is usually reported in the eastern United States; the highest rates occur in Arkansas, Missouri, North Carolina, Oklahoma, and Tennessee. Most cases occur in the spring and summer, when arthropods are more active.

The incubation period of RMSF is 2 to 14 days, although many patients with RMSF do not recall a tick bite. Headache, fever, and myalgias often occur first, followed by a maculopapular rash on the extremities 3 to 5 days after illness onset. The rash occurs in about 90% of patients and usually starts at the wrists and ankles, classically involves the palms and soles (though not always), and spreads rapidly and centripetally to most of the body. **Petechiae** and purpura represent extravasation of blood from blood vessels into the skin because of the cutaneous vasculitis, and skin necrosis or gangrene may occur. RMSF is fatal within 1 to 2 weeks if appropriate therapy is not administered. The clinical syndrome of RMSF may be confused with atypical measles, mononucleosis, disseminated meningococcal or gonococcal infection, other forms of bacterial sepsis,

secondary syphilis, typhoid fever, enteroviral infection, and leptospirosis.

Because culture of these organisms is difficult, diagnosis is made based on clinical presentation and serology. The low bacterial burden in the blood limits the sensitivity of PCR assays. *R. rickettsii* is resistant to many common antimicrobial agents, but the infection is generally successfully treated with doxycycline or chloramphenicol if therapy is given early in the disease course.

Boutonneuse fever, also known as *Mediterranean spotted fever*, is the most severe spotted fever rickettsiosis after RMSF and is caused by *Rickettsia conorii*. It is characterized by the *tache noir* (black spot) or eschar at the site of the tick vector bite. This illness is not endemic in the United States but can be seen in travelers returning from India, Pakistan, Africa, and eastern and southern Europe in areas around the Mediterranean Sea. The skin lesion is the result of endothelial injury in soft tissues with necrosis and perivascular edema. Rickettsialpox, caused by *Rickettsia akari*, is also one of the spotted fever group of rickettsial diseases, but it is transmitted by the bite of mites, rather than ticks, and mice are the reservoir. The organism occurs worldwide but was initially found in New York City. Clinical findings include fever, rash, and eschar. The eschar develops from a papule that vesiculates. The rash spares the palms and soles, is papular and vesicular, and leaves black crusts. These illnesses are treated similarly to RMSF, with doxycycline or chloramphenicol.

Typhus fever is caused by two species of rickettsiae, *R. prowazekii* and *R. typhi*. The former causes epidemic or sylvatic typhus and is transmitted by human body lice, and the latter causes murine typhus and is transmitted by fleas. Epidemics occur in conditions of poor hygiene. Symptoms include fever, headache, myalgias, and a macular erythematous rash. Diagnosis is made serologically, and treatment is with doxycycline.

Scrub typhus, caused by *Orientia tsutsugamushi*, is transmitted via the bite of a chigger (mite larval stage). It is distributed worldwide in rural areas with high grass and brush, particularly in India, China, Southeast Asia, and Russia. An erythematous papule occurs at the site of the bite followed by ulceration and eschar formation. A macular faint rash involving the trunk and extremities often occurs. Systemic symptoms include fever, headache, myalgias, and lymphadenopathy. Serologic testing is typically used for diagnosis, and treatment is with doxycycline.

Leptospirosis

Leptospirosis is a zoonotic disease usually caused by *Leptospira interrogans*, although other *Leptospira* species can cause disease. *Leptospira* species are spiral aerobic spirochetes distinguished from other spirochetes by their hooked ends. The organisms are carried in the renal systems of rodents and other mammals, including dogs and livestock. Humans become infected after direct contact with an infected animal or indirect contact with the urine or tissues of infected animals in vegetation, soil, or water. Leptospirae can remain viable in the environment for months.

The infection is found throughout the world, particularly in tropical and subtropical areas, and the highest incidence in the United States is in Hawaii. Leptospirosis commonly

involves the kidneys, liver, and CNS. It can be a self-limited illness, or it can be fatal with renal and liver failure and pneumonia. The rash of leptospirosis is maculopapular and may become hemorrhagic. Like *Treponema*, *Leptospira* can be seen by darkfield microscopy, but the technique is neither sensitive nor specific and not widely available, and the diagnosis of leptospirosis is usually made serologically. The organisms can be cultured, but culture is a slow and insensitive technique. Penicillin G, ceftriaxone, and doxycycline are generally effective for treatment.

Bartonellosis

The most common forms of human bartonellosis are caused by *Bartonella henselae* and *Bartonella quintana*. *Bartonella* spp. are gram-negative intracellular bacteria transmitted by insects such as phlebotomine sandflies, human body lice, and cat fleas or by animal scratches or bites. They can cause acute and chronic infections and blood vessel proliferation. *B. henselae* is the cause of cat scratch disease (CSD), which is transmitted by cat scratches or bites. Cat fleas may also be a vector for *B. henselae*. The initial lesion of CSD is a papule, pustule, or vesicle, usually on the arm, followed by the development of regional lymphadenopathy associated with fatigue, fever, and malaise. The infection can resolve spontaneously after weeks to months, and antimicrobials generally fail to affect the course. *B. quintana* is transmitted by louse vectors and associated with poor sanitation and hygiene.

Both *Bartonella* spp. can cause bacteremia and endocarditis as well as the main dermatologic disease of bartonellosis, bacillary angiomatosis (BA), which is predominantly seen in HIV-infected persons, although infections have also been described in solid organ transplant recipients. BA is caused by new blood vessel formation in the skin or other organs, such as the liver (known as *peliosis hepatis*). The skin lesions that occur in crops are generally nodular and red or purple and can ulcerate. The size of the lesions ranges from several millimeters to centimeters, and they bleed easily with trauma. They are most prominent on the extensor surfaces of the limbs. The organism may be cultured from skin and subcutaneous lesions or occasionally from blood, but *Bartonella* spp. are fastidious and may require more than 1 week of incubation on specialized media. Diagnosis can also be made by serologic testing, PCR of blood, or histopathologic demonstration of organisms in tissue specimens using Warthin-Starry staining.

Rat bite fever

Two bacterial diseases, which are rare in the United States, are included under the general term *rat bite fever*: streptobacillosis, caused by *Streptobacillus moniliformis*, and spirillosis, caused by *Spirillum minus*. The majority of U.S. and European cases are caused by *Streptobacillus*, whereas *S. minus* infections occur predominantly in Asia. These gram-negative organisms are part of rodent oral biota. Rat bite fever is usually transmitted by bites or scratches from rodents or carnivores that ingest rodents, but streptobacillosis has also occurred after ingestion of food contaminated by rodent excrement. The diseases share clinical and epidemiologic characteristics. An abrupt onset of fever and chills, headache, and muscle pain is followed shortly by a maculopapular or sometimes petechial,

vesicular, or pustular rash that is most marked in the extremities, may become purpuric, and can lead to desquamation. The rash involves the palms and soles, and the illness can mimic RMSF or secondary syphilis. In *S. moniliformis* infection but not *S. minus* infection, joint swelling and pain often occur in association with the rash.

Laboratory confirmation is made by isolation of the causative organism after inoculating material from the primary lesion, lymph node, blood, joint fluid, or pus into culture media or laboratory animals. *Streptobacillus* characteristically forms filaments and beadlike chains on Giemsa or Gram-stained smears, and *S. minus* appears as thick, coiled spiral rods. Serum antibodies may be detected in *S. moniliformis* but not *S. minus* infection, and PCR and MALDI-TOF mass spectrometry have been used for diagnosis of *S. moniliformis* infection. Penicillin G is the treatment of choice for both infections.

Tularemia

Tularemia, also known as *rabbit fever*, is caused by *Francisella tularensis*, a gram-negative coccobacillus. The disease occurs in six forms, including glandular, ulceroglandular, oculoglandular, pneumonic, oropharyngeal, and septicemic or typhoidal. The type and severity of disease depend on the subspecies or strain of infecting bacteria, dose, and route of infection. Transmission can occur through contact with infected animals (especially rabbits and small rodents), contact with contaminated water or soil, or from arthropod bites. The most common form of the disease is ulceroglandular, in which the infection is inoculated directly into the skin after handling infected rabbits, rodents, and other wild animals or, less commonly, domestic animals such as sheep or cats. This form of infection can also develop after the bite of infected fleas, ticks, deerflies, and mosquitoes. It presents as an indolent painless ulcer, often on the hand, accompanied by painful swelling of the regional lymph nodes. Some patients can have nodular lymphangitis. Infection that manifests as lymphadenopathy without report of an ulcer is termed *glandular*. The other forms of tularemia can occur via inoculation of the conjunctiva (oculoglandular) or inhalation of bacteria from the environment (pneumonic). Pulmonary infection may also occur from hematogenous spread from another site of inoculation. All forms may be associated with maculopapular or vesicular rash and erythema nodosum, urticaria, and erythema multiforme.

Diagnosis of tularemia is usually made by demonstrating specific antibodies in the patient's serum, as early as 1 week after onset of symptoms. Standard antibody detection is by indirect immunofluorescence assay or microagglutination methods. Examination of ulcer exudates, lymph node aspirates, and other specimens using a fluorescent antibody test may provide a rapid diagnosis, although the organism is not often seen on Gram stain. PCR methods have been developed to detect *Francisella* DNA in clinical specimens, and MALDI-TOF mass spectrometry analysis can rapidly identify the organism from culture. *Francisella* is fastidious but will grow on chocolate, modified Thayer-Martin, and buffered charcoal-yeast extract agars using material from lesions, blood, or sputum. Laboratory isolation of *Francisella* constitutes a critical value. Great care must be exercised with culturing the organism because of its highly infectious nature. Infection by *Francisella* is a potential occupational hazard for laboratory workers. Streptomycin with or

without tetracycline has been the traditional treatment, but because of the potential for ototoxicity and nephrotoxicity, streptomycin is generally reserved for more serious cases. Doxycycline and fluoroquinolones are effective treatments for uncomplicated cases.

Mycobacteria

Nontuberculous mycobacteria and tuberculosis

As noted earlier in this chapter, NTM can cause localized cutaneous infection, generally as the result of skin inoculation via trauma, sometimes extending to bone and joints (Fig. 33.17). These infections can also become disseminated. This typically occurs in the setting of impaired immune function, as in persons with HIV, organ transplantation, and cancer. Patients with central venous catheters may develop NTM catheter-related infections that can spread hematogenously to various organs, including the skin. These infections are usually the result of rapidly growing mycobacteria including *M. fortuitum*, *M. chelonae*, *M. abscessus* complex, and *M. mucogenicum*. The characteristics of the skin lesions and their treatments are like those noted earlier for localized cutaneous disease caused by NTM.

M. tuberculosis can spread to the skin through the lymphatic system and bloodstream to distant sites and cause a variety of skin manifestations. Hematogenous spread to cutaneous sites leads to nodular swellings of the skin forming tuberculous gummas (skin abscesses) or lupus vulgaris, the most common form of cutaneous tuberculosis. Lupus vulgaris is characterized by sharply circumscribed, red-brown plaques or ulcers of the head and neck. The infection can be progressive and can destroy cartilage of the nose or ears. Lesions may be nodular, scaly, or ulcerated. Skin biopsy and mycobacterial culture often make the diagnosis, although organisms may not be seen in biopsies of lupus vulgaris as they tend to be few. Other forms of cutaneous tuberculosis are the result of hypersensitivity reaction rather than significant skin infection with



Fig. 33.17 Subcutaneous nodules and cellulitis caused by disseminated *Mycobacterium chelonae*.

the organism. For example, erythema induratum of Bazin can develop in patients with extracutaneous tuberculosis, characterized by multiple tender, indurated nodules, typically on the legs. Biopsy of these lesions shows inflammatory changes, including vasculitis and panniculitis, but organisms may not be detected. However, the lesions do respond to antituberculous therapy.

Leprosy

Leprosy, also known as *Hansen disease*, is found in South Asia, Africa, and South America. Cases reported from the United States are typically in immigrants from these endemic areas. In addition, genetic factors appear to influence susceptibility to Hansen disease and the disease manifestations. The classic skin manifestation of *Mycobacterium leprae*, the causative agent of Hansen disease, is a circumscribed, hypopigmented, or less commonly, reddish, hyperpigmented macule (Fig. 33.18). However, lesions can also be papular or nodular, and affected skin may have decreased or no hairs.

Two polar types of Hansen disease occur, tuberculoid or lepromatous, with three recognized gradations occurring between these two extremes. Classification is based on the number and appearance of the skin lesions, nerve involvement, and systemic or mucosal involvement. Tuberculoid leprosy, the mildest form, is characterized by few skin lesions with a paucity of mycobacteria in the lesions and a strong host cell-mediated immune response with extensive lymphocyte infiltration and granulomas. Patients with lepromatous leprosy, the most severe form, have several lesions with many mycobacteria, infiltration of the peripheral nerves, and a poor cell-mediated response with fewer lymphocytes and granulomas. Because the peripheral nerves are infiltrated with organisms and may be injured by the host immune response, there is nerve dysfunction, and the skin lesions are often anesthetic. *M. leprae* has a predilection for the cooler parts of the body, such as the ears and nose. The main mode of spread is via infected respiratory or nasal secretions, although leprosy is not easily transmitted. Acid-fast bacilli and granulomas can be seen in tissue biopsy specimens, but *M. leprae* has not been cultured in vitro, except in live animals. Effective treatment usually involves combination chemotherapy with agents such as rifampin, dapsone, and clofazimine.



Fig. 33.18 Hypopigmented macules of leprosy. (Courtesy Dr. Gail Reid, Loyola University Medical Center, Maywood, IL.)

Fungi

Candidiasis

Skin lesions can be a clue to the diagnosis of invasive forms of *Candida* infection, such as candidemia. An erythematous maculopapular, pustular, or nodular rash may be associated with systemic *Candida* infection. The lesions represent subcutaneous *Candida* abscesses. Skin biopsy will reveal fungal pseudohyphae with inflammatory cells, and *Candida* can be grown from culture of the aspirate or biopsy.

Systemic dimorphic fungi and molds

S. schenckii (see earlier), *Histoplasma capsulatum*, *Blastomyces dermatitidis*, and *Coccidioides immitis* are the most common systemic dimorphic fungi. These fungi often cause disease in healthy hosts, unlike opportunistic fungi, such as *Aspergillus*. They are known as *dimorphic fungi* because they exhibit different morphologies at different temperatures, existing as molds in the environment at 22° to 30° C but as yeasts at body temperature. Within the United States, *Histoplasma*, *Blastomyces*, and *Coccidioides* are distributed in specific geographic areas: *Histoplasma* in the Ohio and Mississippi River Valleys; *Blastomyces* in the Midwest, South Central, and Southeastern United States; and *Coccidioides* in the Southwest.

These three genera usually enter the body via the respiratory tract and primarily cause pneumonia, but all can become systemic and produce secondary skin or mucosal lesions through hematogenous spread. The skin is the most common extrapulmonary site of *Blastomyces* infection. Well-circumscribed tender nodules (Fig. 33.19), ulcers, or plaques can occur anywhere on the body, often on the exposed areas of the head, neck, or extremities. The lesions are typically verrucous and can ulcerate or form abscesses that drain purulent fluid. The skin manifestations of blastomycosis may be mistaken for pyoderma gangrenosum, NTM infections, skin cancer, or other fungal infections.

Skin biopsy reveals neutrophilic and granulomatous inflammation with broad-based budding yeasts with a



Fig. 33.19 Nodular skin lesion caused by *Blastomyces dermatitidis*. (Courtesy Dr. Gail Reid, Loyola University Medical Center, Maywood, IL.)

doubly refractile cell wall, 8 to 15 µm in size. Silver and periodic acid–Schiff stains are helpful in identifying the organisms that will also grow on SDA. In addition, urine and serum antigen assays are available to detect *Blastomyces*. These tests are often positive in disseminated disease. The organisms are sometimes isolated from blood cultures.

Compared with *Blastomyces*, *Histoplasma* is less often associated with skin lesions, but when cutaneous manifestations of histoplasmosis do occur, they can be papular, nodular, plaquelike, or ulcerated, or they may take the form of cellulitis. Disseminated histoplasmosis is an opportunistic infection in persons with AIDS, organ transplant recipients, or those receiving immunosuppressive medications such as tumor necrosis factor α (TNF- α) antagonists (e.g., infliximab) for the treatment of inflammatory diseases such as rheumatoid arthritis or Crohn disease. Cutaneous lesions are not uncommon in this setting and may be accompanied by oral and GI tract ulcers or nodules caused by *Histoplasma*. Sometimes, oral lesions are the only manifestation of histoplasmosis.

The diagnosis is made similarly to that of blastomycosis, using skin biopsy and culture. *Histoplasma* yeasts are smaller than *Blastomyces*, 2 to 4 µm in size, and are thick walled with narrower-based budding. As with blastomycosis, *Histoplasma* urine and serum antigen tests are available to assist in diagnosis, and blood cultures may be positive in disseminated disease.

One of the most common areas of the body to which *Coccidioides* spreads is the skin, which may be the only clinical manifestation of disease. Lesions can be maculopapular, verrucous, ulcerated, or fluctuant abscesses, with a propensity to involve the scalp and face. Diagnostic methods for coccidioidomycosis are like those for histoplasmosis and blastomycosis, namely, tissue biopsy, culture, and antigen testing. *Coccidioides* is highly contagious, so the laboratory should be notified that it might be present when clinical material is sent for culture. In tissue, *Coccidioides* organisms appear as 10- to 80-µm thick-walled spherules that may contain endospores and neutrophilic infiltrates surrounding the spherules. In addition, serum antibody testing for coccidioidomycosis is useful for diagnosis. For all the endemic fungi, systemic antifungal therapy with amphotericin B (for severe disease) or azoles is generally effective.

Disseminated mold infections, including those caused by *Aspergillus*, those that cause mucormycosis (see earlier), and those caused by *Fusarium* are serious infections with high mortality rates. Patients become infected by these fungi via inhalation of fungal spores, ingestion of contaminated food, or skin trauma. They invade blood vessels and often spread, including to the skin. Cutaneous lesions are typically black and necrotic, with eschar formation. These infections generally have a poor prognosis as they tend to occur in severely immunocompromised persons such as bone marrow transplant recipients or persons with acute leukemia.

A diagnosis is made by observing characteristic fungal hyphae on tissue stain and fungal culture of a suspicious skin lesion. In addition, *Fusarium*, unlike other molds, can be recovered in blood cultures. In an immunocompromised patient, the presence of one of these molds, particularly *Fusarium* or one of the agents of mucormycosis, in a culture or tissue stain is a critical value and should be communicated to the patient's health care provider immediately. Amphotericin B or mold-active azoles are used for these infections.

Viral infections

Viruses are intracellular parasites that require a living, nucleated cell to replicate. Viruses can produce localized or disseminated disease and, in some cases, cause chronic infection by evading the host immune system (e.g., HIV, hepatitis C virus). They can cause direct damage to tissues or evoke an immune response that leads to disease manifestations. The viruses commonly associated with skin and soft tissue infections are discussed in this section.

Rubeola

Rubeola, also known as *measles*, is caused by a paramyxovirus (family Paramyxoviridae, genus *Morbillivirus*) and is spread by direct contact with respiratory secretions of infected persons. Measles is one of the most communicable of all infectious diseases, but after vaccination became available in the 1960s, the number of cases in the United States decreased sharply, and the illness was considered controlled in the United States in 2000. However, there has been a recent marked increase in measles cases. In 2019, more than 700 cases occurred in the United States, the largest number in the 21st century. About 70% of those cases were in unvaccinated patients, and almost all were residents of the United States.

After an incubation period of 10 to 14 days, the clinical features of coryza, conjunctivitis, and cough develop, followed by the appearance of Koplik spots. These are small red patches with central bluish-gray specks on the buccal mucosa near the second molars. Shortly thereafter, a maculopapular rash occurs. The rash spreads from the face downward to the trunk and extremities and affects the palms and soles. Patients with measles are most infectious during the late prodromal phase of the illness, when cough and coryza are at their peak. However, the disease is contagious from several days before the rash to several days after its onset. Measles can cause pneumonia or be complicated by secondary bacterial pneumonia, and it may cause meningoencephalitis, particularly in immunocompromised persons.

Viral isolation is difficult because of slow growth of the virus and the limited number of cell types in which it can grow. The diagnosis is usually based on signs and symptoms, but PCR for viral RNA can be performed from a throat, nasal, or posterior nasopharyngeal swab. Confirmation can also be performed via serologic testing for antibody titers.

Rubella

Rubella, also known as *German measles*, is a viral infection of children and adults that resembles measles. It is characterized by fever, rash, and lymphadenopathy. The virus is in the family Togaviridae and the genus *Rubivirus*. Rubella virus is spread in droplets shed from the respiratory secretions of infected persons. The contagious period extends from about 10 days before appearance of the rash to 15 days after onset, but it is maximal 7 days before to 7 days after onset. Many rubella infections are subclinical. For symptomatic persons, a nonspecific maculopapular rash begins on the face and moves down the body, often accompanied by cervical and

occipital lymphadenopathy and, at times, splenomegaly. The disease is clinically milder than measles, although arthritis, myocarditis, and encephalitis can be complications, and serious congenital defects (e.g., cataracts, heart defects, and deafness) can be a consequence when the infection occurs during pregnancy.

Like measles, culture of rubella virus is cumbersome and often not available; therefore diagnosis can be made by PCR detection of the virus from urine, throat, or nasopharyngeal specimens. Diagnosis can also be made by serum antibody testing if there is an increase in antibody titers over several weeks. Congenital rubella infection is often diagnosed by identifying rubella virus nucleic acids by PCR in infant specimens.

Parvovirus B19 infection

Erythema infectiosum, or fifth disease, is one of the common viral **exanthems** of childhood. Most persons encounter this infection at some point—up to 80% of adults are seropositive, depending on the population surveyed. It is caused by human parvovirus B19 (family Parvoviridae, genus *Erythroparvovirus*), which replicates in erythrocyte precursor cells. The primary mode of transmission is thought to be via the respiratory route, although vertical transmission to the fetus can occur during pregnancy, and transmission has occurred through blood product transfusion.

Fifth disease is characterized by a bright red rash of the face (so-called *slapped cheek* appearance) followed in 1 to 4 days by a fine, lacelike rash on the trunk or extremities. The rash may fade quickly, only to recur during the ensuing 2 to 3 weeks on exposure to sunlight or heat. Infection is generally not associated with fever, although mild constitutional symptoms may precede the rash. **Scarlet fever** and rubella are other childhood diseases to be distinguished from fifth disease. Variation of the rash can include the “glove-and-socks” distribution of the skin lesions, which, in this case, tend to be papular, petechial, pustular, or bullous. Complications of parvovirus B19 infection are more common in adults, and some patients can develop a symmetric arthralgia and arthritis. In those with hemolytic disorders and immunosuppressed persons (e.g., those with HIV or organ transplantation), parvovirus B19 can cause marked inhibition of red blood cell formation with extremely low red blood cell counts. Parvovirus B19 infection in pregnancy can lead to hydrops fetalis and miscarriage. Diagnosis is often made by detecting the virus in blood or bone marrow with PCR assay. Serious manifestations of infection are treated with intravenous immunoglobulin (IVIG) and blood transfusion; no antiviral medication is available.

Enteroviral infections

Coxsackieviruses and echoviruses, members of the family Picornaviridae and genus *Enterovirus*, cause a variety of exanthems. Enteroviral infections are more prevalent in the summer and fall months in temperate climates and occur year-round in tropical regions. Most infections occur in children, particularly infants, and mucocutaneous features are also found more commonly in infants and children than in adults. Enteroviruses are predominantly spread via the fecal-oral

route but at times may be spread by respiratory secretions. Most enteroviral infections are asymptomatic or result in a brief febrile illness or upper respiratory tract infection, but coxsackie viruses and echoviruses can cause more serious disease, including aseptic meningitis or encephalitis, acute flaccid paralysis, hemorrhagic conjunctivitis, pericarditis, myocarditis, and pleuritis. Enteroviruses are also the cause of many different exanthems, some of which have oral mucosal involvement. These are generally benign diseases and can resemble other viral causes of rash. They are grouped as rubelliform or morbilliform (resembling rubella or measles), roseoliform (resembling roseola), or herpetiform (resembling herpes) exanthems.

Enteroviruses can also produce petechial or purpuric-type rashes resembling those of meningococcal septicemia. A common rash associated with a number of different enterovirus serotypes is herpetiform hand, foot, and mouth disease (HFMD). HFMD primarily occurs in young children and is characterized by fever, throat pain, and vesicles on the palate, tonsils, and tongue that evolve into painful ulcers. Erythematous papules and vesicles that resemble HSV or varicella-zoster virus (VSV) lesions can also occur on the lateral and plantar aspects of the feet, palms, dorsal fingers, and sometimes the buttocks and genitalia. Herpangina is another form of mucosal disease that can occur, predominantly caused by coxsackieviruses, and is characterized by fever, throat pain, and vesicular lesions on the soft palate and posterior oropharynx. It may be confused with other causes of pharyngitis.

Viral culture of stool, throat, or involved tissue can be performed to make a diagnosis but is rarely performed. Serologic testing demonstrating a fourfold increase in antibody titer can also be used. Most commonly, PCR testing of the oropharynx, stool, or vesicle fluid establishes the diagnosis.

Herpesviridae

There are hundreds of Herpesviridae viruses, although only eight are common causes of human infection. All eight can lead to dermatologic disease, but some, such as VZV and HSV, are associated with more prominent skin findings.

Varicella-zoster viral infections

Varicella infection, or chickenpox, is a common childhood illness acquired by respiratory inhalation of VZV. The skin lesions of primary VZV infection become apparent approximately 2 weeks after initial exposure. The lesions begin as vesicles or small blisters on an erythematous base (“dew drop on a rose petal”). The lesions become pustular or rupture and form eschar over a period of several days. The rash starts on the trunk and face and then spreads to the extremities. A characteristic of chickenpox is the presence of lesions in various stages of development. When primary VZV infection occurs in adults, it tends to be more severe and systemic, involving the lungs, CNS, and liver.

Treatment with the antiviral agent acyclovir is recommended for immunosuppressed individuals and patients with visceral involvement. Routine administration of varicella vaccine to children in the United States has been 98% effective (after two doses) in preventing disease caused by VZV with little waning of immunity over time. The use of

the vaccine has resulted in a decrease in hospitalizations and deaths caused by VZV infection.

VZV, like other members of the family Herpesviridae, produces lifelong latency in the human host. After primary infection, the virus enters peripheral nerves and persists within the dorsal root ganglia. The virus may be reactivated under various conditions, most importantly by immunosuppression associated with age, certain diseases, or drug therapies (e.g., corticosteroids, cytotoxic chemotherapy). With reactivation, the virions move along peripheral sensory nerves of the skin and produce a vesicular eruption in a unilateral dermatomal distribution (Fig. 33.20). The resulting condition is called *herpes zoster* or *shingles*. The vesicles are like those of chickenpox but remain localized along sensory nerves. The disease is generally benign but may lead to postherpetic neuralgia, a syndrome of lingering pain at the site of the healed rash. In addition, when the face is involved, the virus may spread along the ophthalmic branch of the trigeminal nerve to the eye (zoster ophthalmicus), resulting in sight-threatening disease. In immunosuppressed patients, the virus may spread widely to the skin and to internal viscera such as the lungs, meninges, brain, and liver. The Centers for Disease Control and Prevention recommends two doses of the recombinant vaccine Shingrix (GlaxoSmithKline, Brentford, United Kingdom) in persons over 50 years of age to prevent shingles.

The diagnosis of chickenpox or herpes zoster is often made clinically given the characteristic epidemiologic and presenting features but can be confirmed by taking a scraping of the base of a vesicular lesion and performing PCR. Viral culture was once the standard for diagnosis but is slow, labor-intensive, and less sensitive than PCR. Culture may be required in the unusual setting of suspected antiviral resistance and the need for testing antiviral drug susceptibility. Direct fluorescent antibody (DFA) testing is also available to obtain a rapid diagnosis from a tissue scraping. Acyclovir or the orally administered prodrugs valacyclovir and famciclovir are used for the treatment of herpes zoster. Intravenously administered acyclovir is given for severe or disseminated disease.



Fig. 33.20 Hemorrhagic vesicular lesions of herpes zoster. (Courtesy Dr. Gail Reid, Loyola University Medical Center, Maywood, IL.)

Herpes simplex viral infection

HSV types 1 and 2 cause some of the most common skin and mucous membrane infections affecting humans. HSV infections occur after contact of mucous membranes or abraded skin with infected secretions. Initial infection may lead to the characteristic vesicular skin lesions at the site of infection or may be asymptomatic. After penetrating the skin or mucosa, the virus enters nearby neurons and leads to a lifelong latent infection in neuronal ganglia that can reactivate under various conditions, particularly immunosuppression, and cause recurrent disease. Both HSV-1 and HSV-2 can cause oral or genital infection, but HSV-1 more commonly causes orofacial infection, and HSV-2 tends to cause genital infection. An increasing proportion of genital herpes caused by HSV-1 has been noted in industrialized countries. Clinical findings cannot distinguish HSV-1 from HSV-2. Cutaneous HSV lesions are also indistinguishable from those caused by VZV, although HSV skin lesions usually do not follow a dermatomal pattern. In severely immunosuppressed hosts, such as persons with AIDS, hematologic and lymphoreticular malignancies, or organ transplants, a disseminated and life-threatening form of HSV can occur, with diffuse cutaneous lesions and lung, liver, or other visceral organ involvement.

Primary HSV-1 infection may present as severe ulcerative gingivostomatitis and pharyngitis and typically occurs in children younger than 5 years of age. This initial bout of infection is often accompanied by fever and systemic toxicity. Oral vesicles involving the soft palate, buccal mucosa, tongue, lips, and roof and floor of the mouth quickly ulcerate and may coalesce. Primary herpetic gingivostomatitis can resemble bacterial pharyngitis, herpangina caused by coxsackievirus, aphthous stomatitis, erythema multiforme major (Stevens-Johnson syndrome), infectious mononucleosis, and chemotherapy-induced mucositis. Herpes labialis, a condition commonly known as *fever blisters* or *cold sores*, is the most common manifestation of HSV reactivation. Lesions begin as superficial clear vesicles on an erythematous base on the lips or in the oropharynx. The lesions heal spontaneously and may recur in the same area. Recurrent herpes labialis is generally unaccompanied by systemic complaints, in contrast with primary infection.

Other notable cutaneous manifestations of HSV infection include eczema herpeticum, a vesicular eruption that involves areas of chronic eczema or burns and can be quite severe (Fig. 33.21), and erythema multiforme, an acute hypersensitivity reaction that manifests as target-type erythematous skin lesions that may involve mucous membranes. HSV DNA has been identified in biopsies of erythema multiforme skin lesions, and it is thought that HSV is the cause of most cases of recurrent erythema multiforme. Another presentation of cutaneous HSV is herpetic whitlow, or primary herpetic lesions of the finger (Fig. 33.22). This can be caused by HSV-1 or HSV-2. Usually a single digit is involved, with the appearance of one or more deep vesicles that may coalesce. Fever and intense local pain are often present. The condition may be misdiagnosed as bacterial paronychia, and an unnecessary incision may be performed. Recurrent herpetic whitlow can be a difficult occupational problem in medical, paramedical, and dental personnel. Herpetic whitlow in newborns (following finger sucking) and serious disseminated infection are two important neonatal herpes syndromes. Infants are infected during passage through the infected maternal genital tract.

Herpes gladiatorum outbreaks can be seen among wrestlers and are characterized by herpetic lesions of the face, hands, and chest; transmission occurs via skin trauma during wrestling matches.

HSV-2 in adults is transmitted primarily by sexual contact and results in primary and recurrent herpes genitalis and perirectal infections. Initial infection may be accompanied by fever, myalgias, and inguinal lymphadenopathy. Vesicles and ulcerations occur in the genital and/or perirectal regions. Urethritis and meningitis are potential complications. HSV-1 can also cause genital infection via orogenital contact but typically causes fewer recurrences compared with HSV-2.

The diagnosis of HSV infection can be made clinically but should be confirmed through laboratory methods, ideally via PCR of lesion scrapings. Viral culture of a vesicular or ulcerated skin lesion or biopsy of affected tissue may also be performed but is less sensitive for virus detection from ulcers compared with vesicles and for recurrent lesions compared with primary lesions. Both PCR and culture can distinguish HSV-1 from HSV-2. DFA techniques are also available to

rapidly detect HSV antigens in specimen scrapings. Type-specific serologic testing can be performed to ascertain whether a patient has been infected with HSV-1, HSV-2, or both. Acyclovir, valacyclovir, or famciclovir are the drugs of choice for HSV infections requiring treatment. Intravenously administered acyclovir is given for severe manifestations of disease.

Other herpesviruses

Mononucleosis syndromes caused by Epstein-Barr virus (EBV) and cytomegalovirus (CMV) can sometimes be associated with nonspecific rashes, which are usually maculopapular. EBV can also cause petechial or urticarial rashes and may be a trigger for erythema multiforme. The administration of penicillin therapy during mononucleosis often precipitates an immunologic reaction that leads to a pruritic, erythematous, maculopapular rash.

Human herpesvirus 6 (HHV-6) is the major cause of roseola infantum (also known as *exanthem subitum* or *sixth disease*), a febrile syndrome that occurs in infants and children. High-grade fever is present over a period of several days followed by a maculopapular rash that begins on the trunk and spreads outward. The symptoms are usually benign, although febrile seizures may be present. The diagnosis is usually made clinically, but serologic testing can be performed to confirm the diagnosis. The related human herpesvirus 7 (HHV-7) likely causes a minority of exanthem subitum cases. In immunocompetent persons, treatment for mononucleosis syndromes and HHV-6 and HHV-7 infections is supportive.

Kaposi sarcoma herpesvirus (KSHV), or human herpesvirus 8 (HHV-8), is linked to HIV-associated Kaposi sarcoma (KS) and has been found to have a role in the development of other less common malignancies. KSHV is spread mainly through sexual contact among men who have sex with men, although nonsexual transmission also occurs, possibly via saliva. KS is a vascular neoplasm; its cutaneous form is characterized by the appearance of erythematous or purplish nodules and indurated plaques that may be disfiguring (Fig. 33.23). KS may spread to the lungs, GI tract, or other organs. AIDS-related KS may regress in response to reconstitution of the immune system with antiretroviral HIV therapy, but local or systemic chemotherapy is sometimes required for treatment.



Fig. 33.21 Eczema herpeticum caused by herpes simplex virus.



Fig. 33.22 Herpetic whitlow. (Courtesy Dr. Gail Reid, Loyola University Medical Center, Maywood, IL.)

Molluscum contagiosum

Molluscum contagiosum is a common skin disease caused by a poxvirus (*Molluscum contagiosum virus*, the only member of the genus *Molluscipoxvirus*). Small, firm, waxy papules are present, often with umbilicated centers. Lesions are generally 3 to 5 mm in diameter, but occasionally giant lesions may be seen (Fig. 33.24). The virus replicates in the lower layers of the epidermis and extends upward. Molluscum contagiosum is transmitted directly from person to person, in many cases by sexual contact. It can also be spread from one area of the body to another by contact. The disease usually appears on the genitalia, face, or perirectal area. It tends to be a self-limited disease over a period of months and remains benign, although lesions can be removed for cosmetic reasons by curettage, topical chemical agents, laser, or liquid nitrogen cryotherapy.



Fig. 33.23 Large hyperpigmented plaque of Kaposi sarcoma. (Courtesy Dr. Gail Reid, Loyola University Medical Center, Maywood, IL.)



Fig. 33.24 Giant molluscum contagiosum.

Orf and Milker nodule

Parapoxviruses (family Poxviridae), common pathogens of animals, can cause a pustular dermatitis in humans. Two of the viruses that infect humans are **orf virus** from sheep and goats and **milker's nodule virus** from cattle, also known as *pseudocowpox* or *paravaccinia virus*. These viruses are zoonotic occupational diseases of farmers, veterinarians, slaughterhouse workers, and others exposed to infected animals. The skin lesions are identical, usually a solitary papule, pustule, or nodule that can drain and develops central crusting. Lesions can be associated with local lymphadenitis. The lesions are often located on the hands, arms, or face and can be confused with anthrax. Person-to-person spread is rare, and there is no proven treatment, although topical antiviral agents such as

cidodovir and imiquimod have been used. The illness usually resolves in several weeks without scarring.

Human papillomavirus

Papillomaviruses (family Papillomaviridae) cause a variety of skin and mucous membrane lesions that range in severity from benign growths to malignancies. There are over 200 types of human papillomaviruses (HPVs), with more than 40 types that infect the oropharyngeal or genital regions. Most sexually active persons will experience HPV infection at some point in their lifetime, although infection is often transient. Most human infections are asymptomatic or lead to warts or to benign skin or mucosal cell proliferations. Common warts (circumscribed, hyperkeratotic, rough-textured, painless papules ranging in size from several millimeters to large masses), plantar warts (flat hyperkeratotic lesions of the plantar surface of the feet that may be painful), and flat warts (smooth, slightly elevated, usually multiple lesions ranging in size from 1 mm to 1 cm) are typical cutaneous manifestations. They often resolve spontaneously over time. Certain HPV strains preferentially affect the oral cavity and have been linked to oral cancers.

Genital lesions associated with HPV include venereal warts, or *condyloma acuminata*—cauliflower-like fleshy growths usually seen in the moist genital and perianal regions—and flat papillomas of the cervix. Transmission of HPV from mother to infant via infection of the birth canal can lead to laryngeal papillomas on the vocal cords and epiglottis of children. Some forms of cancer (e.g., penile, vaginal, cervical, oral, and anorectal cancers) may be associated with oncogenic types of papillomaviruses (usually HPV types 16 and 18). HPV DNA testing of cervical cells is often performed along with Pap smear testing in women and can improve the detection of early stages of cervical neoplasia. There is currently no formal indication for testing oropharyngeal, penile, or anal specimens for HPV to screen for cancer at these sites. Treatment of warts is generally with topical chemical therapy, cryotherapy, or laser therapy.

Three vaccines are currently licensed to prevent HPV infection and genital cancers, and they vary by the HPV types they target. Since 2016, only Gardasil 9 (Merck, Kenilworth, NJ) is available in the United States. It is indicated for boys and girls starting at 9 years of age and for women and men through 45 years of age to prevent genital, anal, and head and neck cancers caused by specific high-risk HPV types. In women, the vaccine is also indicated to prevent genital warts caused by HPV types 6 and 11.

Alphaviruses

Several alphaviruses in the family Togaviridae (e.g., Chikungunya, O'nyong-nyong, and Ross River viruses) are mosquito-borne and cause clinically indistinguishable syndromes of fever, arthralgia, and rash. The rash typically starts on the face and neck. As it spreads to other areas, it becomes macular or maculopapular and may be pruritic. Accompanying symptoms include headache, severe arthralgias, retro-orbital pain, pharyngitis, and vomiting. Chikungunya fever, the prototypic disease, is found in Africa, India, Southeast Asia, and areas in Europe, and has recently spread to the Caribbean and the Americas. Local transmission has been reported in the United States since 2014. Serologic

testing or blood PCR makes the diagnosis, and there is no licensed therapy.

Hemorrhagic fever viruses

Several viruses of the families Flaviviridae (e.g., yellow fever virus, dengue virus, Zika virus), Arenaviridae (e.g., Junín virus, Lassa virus, and Machupo virus), Hantaviridae (e.g., hantavirus), Nairoviridae (e.g., Crimean-Congo hemorrhagic fever [CCHF] virus), Phenuiviridae (e.g., Rift Valley fever virus), and Filoviridae (e.g., Ebola virus, Marburg virus) may cause a characteristic viral syndrome, including fever, headache, myalgias, arthralgias, nausea and vomiting, abdominal pain, and skin manifestations. Severe bleeding, including nasal or GI bleeding, may also occur with some of these viruses. Bleeding into the skin also occurs and results in petechiae, purpura, and ecchymoses.

These viruses are usually found in the tropics, and many are zoonotic, having a rodent (bunyaviruses, arenaviruses) or bat (filoviruses) reservoir or an arthropod vector (flaviruses, most human bunyaviruses), with humans as accidental hosts. Some viral hemorrhagic fevers such as Ebola, Marburg, Lassa, and CCHF viruses are spread from person to person through direct contact with a symptomatic patient or infected body fluids, and some may be spread through the slaughter or consumption of infected animals. Treatment is generally supportive, although the antiviral ribavirin has been useful in some cases, particularly with Lassa fever and CCHF. Preventive measures include rodent vector control and isolating persons infected with viruses for which person-to-person transmission occurs.

Dengue virus is one of the more common hemorrhagic fever viruses and the most prevalent human arboviral infection. It is endemic to the Caribbean, the Americas, Africa, and Southeast Asia. The mosquito vector of dengue, *Aedes* (usually *A. aegypti*), is also found in the southeastern United States, and small outbreaks of locally acquired dengue fever have occurred in Texas, Florida, and Hawaii. It is an acute illness manifested by fevers, severe headache, and muscle aches, the latter giving rise to its nickname, *breakbone fever*. As the fever disappears, a macular rash occurs, which spares the palms and soles and often involves the neck and face (Fig. 33.25). The rash is sometimes described as white islands in a red sea because patches of skin are spared from the rash. The signs and symptoms overlap with those of other infections such as chikungunya. Primary infection is usually benign, but dengue hemorrhagic fever is a severe manifestation of disease that occurs in persons who have been previously infected with a different serotype of the virus; petechiae and purpura are common, as are thrombocytopenia, major hemorrhage, disseminated intravascular coagulation (DIC), and shock. Nucleic acid detection of viremia via PCR is the diagnostic assay of choice, although viral culture can be performed, and serologic testing can confirm the diagnosis in a recovering patient. Treatment for dengue fever is supportive.

Zika virus was first identified in the Americas in March 2015 during an outbreak in Brazil. Prior to this outbreak, cases were seen primarily in Africa and Asia. Signs and symptoms of Zika virus include fever, nonpurulent conjunctivitis, myalgias, arthralgias, headache, and retro-orbital pain. Macular or papular rash, often pruritic, occurs in 90% of infected patients. Subcutaneous bleeding may also be seen with acute infection. Zika virus spread throughout the Americas between 2015 and



Fig. 33.25 Facial rash of dengue fever. (Courtesy Dr. Gail Reid, Loyola University Medical Center, Maywood, IL.)

2016, including locally acquired cases in Florida. It has been associated with microcephaly as well as other neurologic disorders in neonates born from infected mothers. Diagnosis is made serologically or by blood PCR, and there is no specific therapy.

Severe acute respiratory syndrome–coronavirus-2 (SARS-CoV-2)

In December 2019, a new virus was reported in Wuhan, China. It spread rapidly throughout the world and resulted in the global pandemic known as Coronavirus Disease 2019 (COVID-19). The coronavirus that was isolated from infected persons, SARS-CoV-2, infects the respiratory tract and can cause severe pneumonia, and it has been responsible for millions of global deaths. COVID-19 also affects many extrapulmonary organ systems, including the skin. There are a variety of dermatologic manifestations that have been described in patients with COVID-19, including nonspecific maculopapular exanthems, urticaria, erythema multiforme, purpura, and a varicella-like papillovesicular rash. Livedo reticularis, a symmetric lacy pattern of red-blue skin discoloration, may also occur. Chillblain-like lesions (“COVID-toes”) have been noted as a characteristic finding; they typically involve the feet and less commonly the hands. They manifest as painful violaceous plaques and nodules. There is no specific treatment for the skin complications of COVID-19, although topical steroids have been used for certain manifestations such as urticaria.

Parasitic infections

There are three main categories of human parasitic infections: helminthic, protozoal, and ectoparasitic. These infections are usually encountered in tropical and subtropical regions and cause an enormous burden of disease in developing countries. Helminths (derived from the Greek term for “worms”) are

multicellular organisms whose adult stages are typically visible to the naked eye. They are extremely prevalent worldwide, particularly in warm, under-resourced areas of the world and include flatworms (trematodes) and roundworms (nematodes). Protozoa are unicellular organisms that can be free-living or parasitic, and they can infect the blood of humans by arthropod vectors. **Ectoparasites** are organisms that attach to and penetrate the skin and live there, rather than inside the human body. Ectoparasites include fleas, lice, ticks, and mites. Many of these parasitic infections have major or minor dermatologic manifestations as part of the illnesses they cause.

Helminths

Schistosomiasis

Schistosomes are trematode flatworms (flukes). Dermatitis caused by schistosomes is usually seen when the infective larvae of bird and nonhuman mammal schistosomes infect humans. Free-living larvae, or cercariae, that have developed in snails penetrate the skin of humans bathing or swimming in infected waters. These nonhuman schistosomes do not mature in humans and die in the skin. The immune response to the organisms results in a hypersensitivity reaction manifesting as an intensely pruritic, papular eruption known as *cercarial dermatitis*, or *swimmer's itch*. There are many different species of schistosomes that can cause dermatitis depending on the geographic location and host animal. Such infections can be prevalent among bathers in lakes in many parts of the world, including the Great Lakes region of North America and certain coastal beaches. The diagnosis is usually made clinically, although the rash may resemble insect bites or dermatitis as a result of bacteria or allergens. The dermatitis is self-limited. Acute schistosomiasis caused by human schistosomes (Katayama fever) is sometimes associated with a similar pruritic rash related to skin penetration by cercariae. This disease is encountered in Asia, Africa, the Middle East, and South America.

Strongyloides infection

The intestinal helminth *Strongyloides stercoralis* is a nematode found in Southeast Asia, Latin America, Africa, and areas in the southeastern United States. Free-living larvae within soil contaminated by human feces penetrate the skin of humans and may cause a transient dermatitis as they migrate to the vasculature on their way to the lung and then GI tract. Other modes of transmission include solid organ transplantation and oral inoculation (e.g., oral-anal intercourse). In chronic infection, adult *Strongyloides* worms reside in the large intestine and produce larvae that can transform before excretion and autoinfect the host by penetrating the intestine or perianal skin, with the latter resulting in a rash known as *larva currens*. The rash is characteristically migratory (larvae can move 2 to 4 inches per hour), serpiginous, and pruritic. Urticaria may appear, and the buttocks, groin, and thighs are often involved given that the larvae penetrate the skin in the perianal area. *Strongyloides* infection may persist for decades asymptotically or as a mildly symptomatic infection but can evolve into hyperinfection (accelerated autoinfection) and severe disease with dissemination. This is usually seen in persons with immunocompromise. The antiparasitic medication ivermectin is effective treatment.

Filariasis

Filariasis is a nematode infection of tropical and subtropical regions that is transmitted to humans by insect vectors. It is characterized by the involved area of the body: lymphatic filariasis and subcutaneous filariasis (discussed later) and serous cavity filariasis caused by *Mansonella* spp. (not discussed further). These infections can be very disfiguring and debilitating, and this section will focus on the worms that cause most of the lymphatic and subcutaneous forms of disease.

Wuchereria bancrofti, *Brugia malayi*, and *Brugia timori* cause lymphatic filariasis. Larval forms are transmitted by mosquito bites, and they transform into adult worms that reside in the lymph nodes and lymphatic vessels of the legs and male genitalia. Adult worms generally live for 5 to 8 years. *W. bancrofti* is responsible for 90% of cases of lymphatic filariasis and is found in tropical areas worldwide. *B. malayi* is found in Southeast Asia, and *B. timori* is found in southeastern Indonesia.

Lymphatic filariasis often occurs in childhood and is typically asymptomatic for many years, but a minority of patients experience significant obstruction of the lymphatic vessels in the lower extremities and genital region, with resulting so-called *elephantiasis* (Fig. 33.26). Fever and inflammation of associated lymph nodes can occur and may be the result of secondary bacterial or fungal infection; these secondary infections are facilitated by the compromised lymphatic function. The parasites can be found in the bloodstream and are identified by Giemsa or hematoxylin and eosin stain of blood smears that should be obtained at night when parasitemia is greater. More sensitive filarial antigen detection and PCR assays are also available. Diethylcarbamazine is effective in killing microfilariae and the adult-stage parasites and can decrease lymphatic obstruction. Doxycycline also has activity against adult worms; it kills *Wolbachia*, rickettsia-like bacteria that live in symbiosis with adult worms and are required for their survival.

Onchocerca volvulus is transmitted by blackflies and causes subcutaneous nodules and river blindness. Onchocerciasis is a chronic nonfatal disease found in sub-Saharan Africa, Brazil, Venezuela, and Yemen. After transmission by blackflies, the larvae develop into adult worms in subcutaneous tissues, particularly over bony prominences in the head and shoulders, hips, and lower extremities. These subdermal nodules are known as *onchocercomata* (Fig. 33.27). The adult worms live for up to 15 years and shed thousands of microfilariae (larvae), which migrate through the skin and have an affinity for the eye. The most important manifestation of onchocerciasis is *river blindness*, so named because the blackfly vector breeds in fast-flowing rivers or streams, and transmission is highest in rural villages near rivers. River blindness results from larval infiltration of the eye. An inflammatory response occurs on death of the larvae, leading to visual disturbances and blindness. The migration of microfilariae through the skin causes an intensely itchy chronic papular rash and skin edema. Depigmentation or thickening of the skin can occur over time (leopard skin).

Laboratory diagnosis of the cutaneous disease can be made by superficial skin biopsies (skin snips), with specimens usually taken from the skin over the iliac crest or scapulae. Microfilariae can then be demonstrated by microscopic examination of the tissue, although the sensitivity of this method may not be as high as serologic testing or urine testing for filarial antigens. PCR on tissue can be performed if microfilariae are not seen. If cutaneous nodules are present, they can be excised to confirm the presence of adult worms.



Fig. 33.26 Disfiguring lower extremity swelling and marked thickening of the skin caused by lymphatic filariasis (sometimes referred to as elephantiasis). (Courtesy Dr. Gail Reid, Loyola University Medical Center, Maywood, IL.)

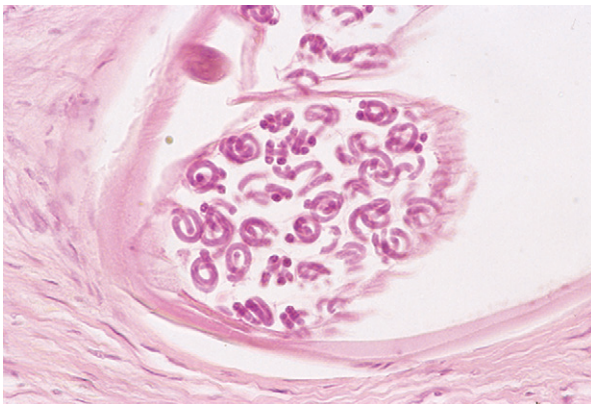


Fig. 33.27 Tissue cross-section of a nodule containing *Onchocerca volvulus* microfilariae (hematoxylin and eosin stain, $\times 400$).

Ivermectin kills microfilariae but has a limited effect on adult worms. Because it can therefore control the disease but may not cure it, ivermectin is often given as an initial single dose, with periodic retreatment on a quarterly or yearly basis. This can reduce morbidity and interrupt transmission of the parasite. Doxycycline also appears effective based on its anti-*Wolbachia* activity. *Wolbachia* is an endosymbiotic rickettsia-like bacterium that appears to be required for *O. volvulus* adult worm survival and for embryogenesis.

Dracunculiasis

Dracunculus medinensis, a nematode found in remote areas of Africa, causes guinea worm disease or dracunculiasis. The World Health Organization launched an eradication program in 1980 that has nearly eliminated the disease. The parasite infects humans who drink pond or stagnant river water contaminated with the intermediate host, a copepod or “water-flea” (genus *Cyclops*) that contains guinea worm larvae. After ingestion, the larvae migrate to the small intestine, where they penetrate the intestinal wall and reside in the peritoneal and retroperitoneal

spaces. The worms mature, and female worms migrate to the skin, particularly the legs, where they cause painful blisters and can emerge through the skin. Worm tracks in the skin can develop cellulitis resulting from secondary bacterial infection. The diagnosis is usually obvious when female worms emerge from the skin to release larvae. Treatment involves slow manual extraction of the worm that can be aided by cooling the skin with ice. There is no effective drug therapy.

Hookworm infection

The human hookworms include the nematode species, *Ancylostoma duodenale* and *Necator americanus*, as well as the more recently described *Ancylostoma ceylanicum*, a zoonosis transmissible to humans. These nematode infections vary in geographic location by genus and species but occur predominantly in warm, moist climates and in areas with poor sanitation. Regions of Africa, Asia, the Middle East, and the Americas are endemic for hookworm infection. Eggs are shed into the environment, and infective larvae develop in soil. As with *Strongyloides* infection, walking barefoot or playing on the ground are risk factors. The larvae can quickly penetrate human skin and cause an erythematous, itchy and papular dermatitis known as *ground itch*, particularly in those previously exposed to hookworm. The larvae then pass through the bloodstream into the lungs, are eventually swallowed, and then travel to the intestine, where they develop into adults and remain.

Larvae of the dog and cat hookworms, *Ancylostoma caninum* and *Ancylostoma braziliense*, respectively, can also penetrate the skin of humans but cannot develop further, as humans are accidental hosts. This results in a self-limited dermatitis known as *cutaneous larva migrans* (CLM), or *creeping eruption*. The dermatitis is characterized by larval migration in the skin, with associated serpiginous, elevated tunnels, and indurated, intensely itchy papules and even bullae. The skin lesions can become superinfected with bacteria, like staphylococci and streptococci. The disease is often seen in children who play in sandboxes frequented by cats and dogs, and it is an occupational hazard of workers who crawl or work in areas with damp sandy soil contaminated by dog or cat feces. Spontaneous cure is the rule, although CLM can persist for months or occasionally years. The diagnosis of CLM can be made clinically. Human hookworm infection is diagnosed by microscopic detection of eggs in stool samples. Albendazole and ivermectin are effective treatments for hookworm infection; thiabendazole may also be effective topically for CLM.

Leishmaniasis

Few protozoal infections cause significant skin infection, but *Leishmania* causes a broad spectrum of disease, with cutaneous leishmaniasis being the most common manifestation. Visceral leishmaniasis, known as *kala-azar*, is a severe form of disease seen with certain species (*L. infantum* and *L. donovani*) in which dissemination of parasites to the bone marrow, liver, spleen, or other organs occurs. *Leishmania* is endemic throughout Latin America, Asia, the Middle East, and southern Europe, and in the United States, south-central Texas is an area of endemicity. *Leishmania* is maintained in mammal reservoirs (dogs, rodents, humans) and is transmitted by the bite of sandflies. Human-to-human transmission from mother to child or through transfusion has also occurred.

Most *Leishmania* infections are limited to the skin and adjacent lymph nodes. Cutaneous leishmaniasis develops days to months after the bite of an infected sandfly, often on the head or extremities, and starts as a small, painless papule that enlarges and may ulcerate (Fig. 33.28). Some lesions remain smooth or become hyperkeratotic, and the appearance of the lesions can differ according to the infecting *Leishmania* spp. The lesions often self-resolve in 3 to 18 months, but local dissemination of infection can occur, with development of additional skin lesions in a sporotrichoid or nodular lymphangitis pattern that can enlarge over years. The lesions can mimic a wide variety of other infectious and non-infectious diseases. Immunocompromised patients may have diffuse lesions that can resemble lepromatous leprosy. Mucocutaneous disease can present as destruction of the lips, palate, and nasal septum. Disfiguring scarring and secondary infection can complicate mucocutaneous leishmaniasis.

Identifying *Leishmania* amastigotes (Leishman-Donovan bodies) in a scraping or biopsy of a skin lesion makes the diagnosis; culture and/or PCR assay can also be performed on the tissue. The organism is fastidious, and Novy-MacNeal-Nicolle medium can be used for culture. Serologic testing is also available. As noted earlier, spontaneous cure of cutaneous lesions is typical over a period of months. Treatment can be given to speed healing and prevent scarring, dissemination, and relapse. Pentavalent antimonial compounds (e.g., sodium stibogluconate, meglumine antimoniate) are the most active drugs but are relatively toxic and expensive. Depending on the species of *Leishmania*, fluconazole, ketoconazole, amphotericin, miltefosine, and pentamidine may have activity.

Ectoparasites

Many ectoparasites may cause human infection, including lice (pediculosis), mites (scabies; Fig. 33.29), fleas, flies, ticks, chiggers, and bedbugs. Ectoparasites attach to or burrow in the skin and can remain there for weeks to months. The dermatologic features of these infestations include severe itching and the formation of papules, vesicles, nodules, linear burrows, and excoriations of the skin and scalp. *Sarcoptes scabiei* var *hominis*, the mite that causes scabies in humans, is too small to be seen by the naked eye and burrows in the stratum corneum, laying eggs that hatch and mature in 3 to 4 days. The rash frequently



Fig. 33.28 Facial ulceration caused by *Leishmania* infection.

occurs in multiple skin sites, with an average of 12 mites on an infected individual, predominantly affecting the hands between the fingers, genital region, waist, wrists, elbows, and ankles. The mite avoids areas with a high density of sebaceous glands. Short, linear burrow tracks are classic, but excoriated papules, hyperkeratotic papules, nodules, or plaques may be seen. Patients complain of generalized itching, which can be severe. Crusted (Norwegian) scabies is a particularly severe infestation that occurs in immunocompromised individuals and causes a psoriasis-type dermatitis (Fig. 33.30).

Scrapings of affected areas of skin with visualization of mites, eggs, or feces by microscopy confirms the diagnosis. Skin biopsy can also be performed, with the burrow seen in the stratum corneum. Treatment consists of topically administered lindane, crotamiton, or permethrin. Orally administered ivermectin is usually given for Norwegian scabies or can be considered for patients who fail topical therapy. Treatment is also given to household and sexual contacts, even if asymptomatic.

Lice are blood-sucking insects. Different types of lice cause infestation of different parts of the body; *Pediculus humanus capitis*, *Pediculus humanus corporis*, and *Phthirus pubis* cause head, body, and pubic infestations, respectively. Lice infestations are also known as *pediculosis* and *phthiriasis*. Body and head lice are similar in appearance: 2 to 3 mm in length, 3 pairs of legs, and tan-gray in color. Lice are transmitted by direct head-to-head contact, with fomites such as brushes or combs less important in transmission because of the short lifespan of lice removed from the body. Transmission of body lice is facilitated by poor hygiene and, as noted earlier, body lice can transmit infection such as typhus, *B. quintana*, and relapsing fever. Intense itching and local erythema, papules, and excoriations of the skin characterize the infection.

The diagnosis is made by identifying live lice (Fig. 33.31) and nits (eggs), although lice move quickly and avoid light. Topically administered permethrin, malathion, and lindane have traditionally been used, although lindane can be associated with neurotoxicity, and resistance to permethrin, the most widely used head louse agent, has been noted. Anthelmintics such as ivermectin (oral or topical administration) or albendazole may be given for severe infestations.

Immune- or toxin-mediated dermatologic manifestations of infectious agents

As described earlier, many different local and systemic infections caused by bacteria, fungi, viruses, and parasites produce diseases of the skin. Skin lesions resulting from direct cutaneous infection and those resulting from hematogenous spread to the skin have been described. Additional dermatologic manifestations of infection can result from the body's immune response to the infecting organism or from toxin production by the organism.

Immune-mediated cutaneous disease

Disseminated intravascular coagulation

Dysregulation of the blood coagulation system frequently occurs during sepsis from any cause and, when severe, can lead

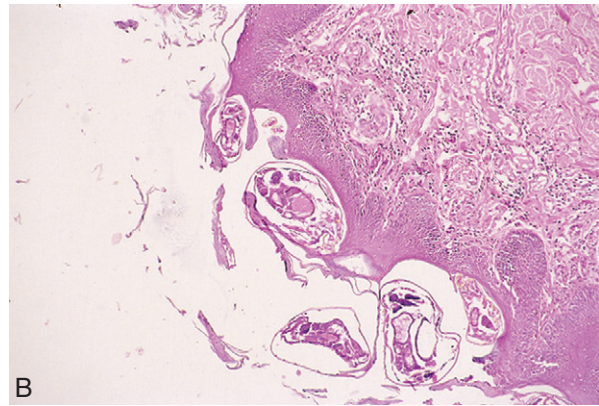
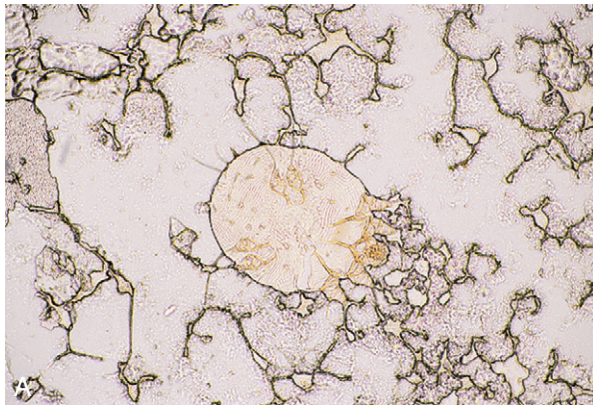


Fig. 33.29 A, *Sarcophaga scabiei* adult showing short legs and conical spines (unstained, $\times 400$). B, Tissue cross-section of scabies lesion showing larvae burrowed into the epidermal layer of the skin (hematoxylin and eosin stain, $\times 400$).



Fig. 33.30 Thick, scaly plaques of Norwegian scabies. (Courtesy Dr. Gail Reid, Loyola University Medical Center, Maywood, IL.)



Fig. 33.31 Parasite, pubic hair from surgical patient, unstained mount ($\times 400$). Three pairs of legs are identified with characteristic claws at the tips. Morphology consistent with *Phthirus pubis* (crab louse). Impression: louse infestation.

to DIC that manifests as bleeding and thrombosis. Important mediators in this process are macrophages and neutrophils; the former recognize invading pathogens and initiate the coagulation cascade, and activated neutrophils release proinflammatory substances leading to neutrophil adhesion, platelet aggregation, and damage to endothelial cells. Leukocyte-platelet aggregation and fibrin deposition in blood vessels is the hallmark of

sepsis-associated DIC. Damaged cells then release other factors that are procoagulants. Thrombi develop in organs such as the liver, lungs, brain, and kidneys, resulting in petechiae of the skin (Fig. 33.32), thrombocytopenia, bleeding, and multiorgan dysfunction. Bacterial sepsis, such as that caused by *Neisseria meningitidis*, streptococci, or enteric gram-negative organisms, is the most common setting in which to encounter infection-related DIC, but it can occur with other types of pathogens, such as the hemorrhagic fever viruses.

COVID-19 is often associated with thrombotic events via activation of the coagulation cascade, intravascular inflammation, and endothelial damage, similar to DIC. However, thrombocytopenia is less often seen in COVID-19, at least early in infection, and prolongation of prothrombin time (PT) is usually minimal. When thrombocytopenia and prolonged PT do occur in patients with COVID-19, patient outcomes appear to be poorer. The mechanisms for COVID-19-induced coagulopathy are still under investigation.

An additional skin manifestation of DIC is purpura fulminans. This syndrome has typically been associated with meningococcemia, but it has also been linked to septicemia with *S. aureus*, *Streptococcus pneumoniae*, and *Haemophilus influenzae*. It is characterized by rapidly developing skin hemorrhage, skin necrosis, and peripheral gangrene accompanied by shock syndrome. Death or amputation often results.

Vasculitis

Many types of infections can trigger direct or indirect immune-mediated damage to small blood vessels in dermal tissues, resulting in palpable purpura, a vasculitic skin lesion typically involving the lower extremities. Infection causes about 20% of cutaneous vasculitis, and it typically results from circulating immune complexes (formed by microbial antigens bound to antibodies) that become trapped in the small vessels of the skin. Cutaneous vasculitis has been associated with bacterial, fungal, viral, protozoal, and helminthic infections. Hepatitis B virus, hepatitis C virus, adenovirus, parvovirus, herpesviruses, streptococci, staphylococci (Fig. 33.33), *Legionella*, rickettsiae, and *Yersinia* are some of the organisms more commonly described as causing vasculitis. Hepatitis C virus can also be associated with a cutaneous vasculitis caused by the production of cryoglobulins, immunoglobulins that precipitate in cold temperatures.



Fig. 33.32 Petechial lesions in meningococcemia.

Infection-related, immune complex-induced cutaneous disease is often seen in endocarditis, with cutaneous purpura the most common skin manifestation. In addition, painful, small skin nodules located on the pads of the fingers and toes and on the thenar eminence, known as *Osler nodes*, may be seen. Traditionally they were thought to be caused by the deposition of immune complexes in soft tissues, although it has also been reported that they are actually microabscesses from small emboli to the skin, similar to Janeway lesions. Janeway lesions are flat, painless hemorrhagic macules located on the palms and soles. Bacteria can be cultured from biopsy specimens from Janeway lesions and have been isolated from Osler nodes. Staphylococci and streptococci are typically associated with these skin manifestations, although gram-negative bacteria and *Candida* may produce similar lesions.

Toxin-mediated cutaneous disease

Certain bacteria, particularly staphylococci and streptococci, can produce toxins that affect the skin, resulting in distinct clinical syndromes. These infections may start as primary skin and soft tissue infections or may initially affect another site, with subsequent involvement of the skin caused by the effects of circulating toxin.

Staphylococcal scalded skin syndrome

Certain strains of *S. aureus* can produce exfoliative toxins that result in staphylococcal scalded skin syndrome (SSSS), a blistering skin condition most commonly seen in children younger than 5 years of age. SSSS is characterized by fever, skin tenderness, and a scarlatiniform rash (resembling the rash of scarlet fever; see later), followed by extensive formation of bullae and exfoliation like that seen in burn patients. SSSS sometimes occurs in adults, particularly those with underlying illness such as renal disease, cancer, IV drug use, HIV, or diabetes mellitus. The toxins act exclusively on the stratum granulosum of the epidermis and thus do not affect mucosal tissues. The Nikolsky sign, separation of the epidermal layer on gentle stroking, is characteristic. Large flaccid blisters form and rupture, causing the skin to denude and peel off in sheets, leaving areas of bright red underlying skin exposed. Secondary bacterial infection and fluid loss can occur, but the skin generally heals without scarring. Nosocomial epidemics



Fig. 33.33 Hemorrhagic vasculitic lesions of *Staphylococcus aureus* endocarditis.

of SSSS have been reported in newborn nurseries. SSSS treatment involves the administration of antimicrobial agents, hydration, and local wound care.

Toxic shock syndrome

Cutaneous desquamation occurs in **toxic shock syndrome** (TSS) caused by the production of staphylococcal exotoxins. These include a unique group of exotoxins that are referred to as *superantigens* because of their ability to cause widespread non-antigen-specific activation of T lymphocytes. Superantigens include toxic shock syndrome toxin 1 (TSST-1), staphylococcal enterotoxins, and streptococcal pyrogenic exotoxins (SPEs). The activation produced by these superantigens results in the rapid release of cytokines by lymphocytes and macrophages. Originally described in children with fever, sore throat, scarlatiniform rash, and desquamation by Todd in 1978, TSS subsequently gained notoriety in 1980 when a number of cases were reported among menstruating women who had used superabsorbent tampons. The tampons apparently provided an environment favorable for TSST-1 production.

Nonmenstrual TSS can occur in many different settings. It has been described in postpartum, after influenza infection, and in association with surgical wound infections and contaminated nasal packing in patients with nosebleeds. Menstrual and nonmenstrual TSS have similar clinical presentations; a diffuse sunburn-like erythroderma appears early in the course and is accompanied by fever, hypotension, and evidence of multiorgan dysfunction. Desquamation of the skin, especially on the palms and soles, occurs during the convalescent stage of the illness. Blood cultures may be positive for *S. aureus*, and staphylococci may be cultured from the initial site of infection. Treatment includes antimicrobial therapy and supportive care with hydration, vasopressors, and debridement of any infected tissue. IVIG may be helpful, presumably through neutralizing the activity of superantigens. Recurrences have been described in as many as 30% to 40% of cases.

Streptococcal TSS caused by GAS (*S. pyogenes*) was increasingly noted in the late 1980s and early 1990s, with the resurgence of invasive and complicated streptococcal infections. Like its staphylococcal counterpart, streptococcal TSS may occur whenever exotoxin-producing strains of GAS infect or

colonize the skin or mucous membranes, particularly strains producing SPEs, although other exotoxins have been implicated. Most streptococcal TSS cases have been in young, otherwise healthy adults, and it is thought that lack of protective immunity is a risk factor. The portal of entry is typically the skin with cellulitis that progresses rapidly, although many invasive streptococcal infections do not have a recognized portal of entry. The subsequent clinical signs and symptoms of GAS TSS are like those of staphylococcal TSS. Blood cultures are more often positive in streptococcal TSS than in staphylococcal TSS. Treatment is with antimicrobial therapy, debridement of infected tissues, and supportive measures. IVIG may be a useful adjunctive therapy.

Scarlet fever

Scarlet fever is a form of GAS disease that can occur when the infecting strain produces SPEs. It occurs mostly in children, concomitantly with pharyngeal infection, although it can also be seen with infections at other sites. Fever is typically present, and the rash starts on the chest and spreads outward. The red, sandpaper-textured rash is often better felt than seen and appears most often on the neck and chest and in skin folds. Typically, the rash does not involve the face, but there is a flushing of the skin, with circumoral pallor, and the patient may have a strawberry tongue (a bright red tongue with dots of white papillae). During convalescence, desquamation of the skin occurs, especially on the hands and feet. The diagnosis is usually made clinically; treatment is with antimicrobials such as penicillins, macrolides, or cephalosporins.

Laboratory diagnosis

A variety of diagnostic methods may be helpful in determining the cause of skin and soft tissue infections. Frequently, primary care providers will swab the surface of broken or ulcerated skin for Gram stain and culture. However, this technique provides little clinically useful information. Swabs of surface wounds or skin are likely to yield colonizing or contaminating bacteria, and there is a lack of correlation between surface colonization and below-the-surface infection. Swabs may also lead to false-negative Gram-stained smears and cultures for several reasons: they may not absorb enough material for culture, organisms may adhere to the swabs rather than being released into culture media, and bacteria do not survive as well on a swab as they do within fluid or a tissue sample. Therefore deep aspirates or biopsies of involved tissue are generally much more informative. For example, if pustules or vesicles are present, the roof or crust should be removed with a sterile blade, and any pus or exudate should be Gram stained and cultured. It is possible to obtain a specimen in a patient with cellulitis by injecting a small amount of preservative-free, physiologic saline into the advancing margin of the affected skin, aspirating it, and then culturing the fluid that is withdrawn. Alternatively, a punch biopsy of the advancing margin can be performed and submitted for Gram stain and culture.

In addition to smear examination and culture of the affected site, exuded pus should always be examined for the

presence of granules and branching filaments suggestive of infection by actinomycetes or fungi. Smears prepared from exudative material and subsequently Gram stained may show the presence of inflammatory cells and characteristic morphologic bacterial or fungal forms that may lead to an initial diagnosis. However, examination of a Gram-stained smear is relatively insensitive for detecting bacteria, and culture is still the best clinically available method of diagnosing cutaneous infections. It is also important to note that organisms may not appear the same on a Gram-stained smear of tissue as they do in pure culture; staphylococci, for example, may not be found in clusters, and streptococci may not form chains.

In addition to Gram stain, it may be appropriate to perform a wet mount with the addition of potassium hydroxide to enhance the appearance of fungi that may be present. Calcofluor white stain may also be used when fungal infections are suspected; yeasts and molds fluoresce and are more readily seen. In addition, if mycobacterial disease is suspected, a Ziehl-Neelsen or Kinyoun acid-fast stain may be performed. Fluorescent stains (rhodamine and auramine dyes) are also available that bind mycobacterial cell walls and are a more sensitive method of staining mycobacteria. To identify *Nocardia* species, a modified acid-fast stain can be performed, using a weaker decolorizing agent than the acid-fast stains. As noted in the “Herpesviridae” section earlier in this chapter, certain viral pathogens such as HSV and VZV can be detected rapidly by obtaining scrapings of new vesicular or ulcerated lesions and performing a DFA test on the specimen to detect viral antigens.

Infectious agents are recovered in routine culture using primary nonselective media, such as blood and chocolate agars, and selective media. MacConkey agar is a selective medium designed to culture nonfastidious gram-negative bacilli and identify those that ferment lactose; phenylethyl alcohol and colistin–nalidixic acid are selective media that preferentially grow gram-positive organisms. For the growth of anaerobic organisms, samples must be collected from infected tissue and transported using an anaerobic transport medium to maximize recovery. Because many anaerobic infections are polymicrobial, samples must be inoculated on culture media that are selective for gram-positive and gram-negative bacteria. Ranges of pH, temperature, oxygen levels, and nutrients are required for the growth of various types of organisms. Therefore communication between the primary care provider and microbiology laboratory is essential for optimizing the information obtained from a clinical specimen.

Case check 33.3

In necrotizing soft tissue infections, cultures are best obtained from purulent discharge or deep tissue biopsy samples rather than by swabbing the surface of a wound. Gram-stained smears from infected tissue in diabetic soft tissue infections may show inflammatory cells and mixed gram-positive and gram-negative bacterial populations. Collection of pus and tissue specimens for culture from diabetic foot infections should also allow the recovery of anaerobic bacteria, which are often present. The microbiology laboratory can identify polymicrobial infections that are often present in diabetic wounds by using various culture media.

Once organisms are growing in culture, identification of isolates is performed using a variety of methods, some of which may be automated. A Gram stain provides the microscopic characteristics of bacterial organisms growing in culture, and further information is obtained from the appearance of the bacterial colonies on laboratory media. Biochemical tests can help identify the organism, while MALDI-TOF mass spectrometry has been increasingly employed in many laboratories. This technique takes advantage of the unique protein profiles of organisms. Assayed sample results are compared with a database for identification. MALDI-TOF mass spectrometry is more accurate than conventional biochemical phenotypic testing traditionally used in clinical microbiology laboratories and can give results in minutes.

Antimicrobial susceptibility testing is subsequently performed on isolates when the susceptibility cannot reliably be predicted based on the organism's identity. For example, GAS have been uniformly susceptible to penicillin, so antimicrobial testing of these organisms is generally presumed unnecessary. Standards have been published and are updated regularly on the performance and interpretation of antimicrobial susceptibility testing based on the correlation of testing results with clinical outcomes. In some cases, there are inadequate data on which to define the drug concentrations at which certain organisms are susceptible to various antimicrobials, particularly for unusual or fastidious organisms.

Other media and techniques are used to culture nonbacterial organisms. *Candida* spp. will grow on blood agar plates, but SDA is a selective fungal medium that has an acidic pH, which is preferred for optimizing fungal growth and suppresses the growth of some bacteria. Lowenstein-Jensen and Middlebrook media are solid media that support the growth of mycobacteria. Certain mycobacteria such as *M. chelonae* and *M. abscessus* grow quickly in culture, but more slowly growing species such as *M. tuberculosis* may take as long as 8 weeks to grow. Studies have demonstrated that mycobacteria grow more quickly in liquid media, allowing the detection of *M. tuberculosis* in as early 1 to 2 weeks.

Nucleic acid probes are available for several of the mycobacterial species, including *M. tuberculosis*, and these can make an identification to the species level once growth is present on a solid medium. Nucleic acid amplification tests (NAATs) are now playing a significant role in the diagnosis of tuberculosis. Commercially available NAAT assays can identify *M. tuberculosis* directly from sputum with a sensitivity that is greater than staining for the bacilli but lower than culture. NAATs can also identify mutations that confer resistance to first-line mycobacterial antibiotics such as isoniazid and rifampin.

Because viruses are intracellular pathogens, they cannot be cultured using the same techniques as for bacteria and fungi and require living cells for isolation (cell culture). If viral culture is desired, specimens should be transported in a special viral transport medium that contains antimicrobial agents to suppress the growth of nonviral organisms. After inoculation of the specimen into a cell culture, the presence of virus is revealed by the observation of a cytopathic effect or a lysing of the cells and separation from the tissue culture substrate. The identity of the virus can then be confirmed by the addition of specific fluorescent antibodies directed against various

viruses or related immunoassay techniques. Because of the cost, technical expertise, and time, most clinical laboratories have replaced cell culture methods with antigen detection assays and NAATs.

Several other non-culture-based techniques for the detection of pathogens that may cause skin infection exist. These include urine antigen detection (e.g., for systemic fungi); serum antibody tests for bacteria, viruses, and parasites; and PCR assays on blood or tissue for the detection of a great variety of bacteria, fungi, and viruses. In addition, multiplex PCR assays are available to simultaneously detect a panel of potential pathogens in blood, respiratory specimens, or stool. For example, the BioFire FilmArray blood culture identification 2 (BCID2) panel (BioFire Diagnostics) extracts nucleic acids from the blood, uses PCR to amplify nucleic acids, and tests for a variety of bacteria and fungi in one test, with results in approximately 1 hour. Pathogens such as *Listeria monocytogenes*, *S. aureus*, *S. pyogenes*, *E. coli*, *P. aeruginosa*, and *Candida* spp. can be detected by this assay. The BCID2 panel can also detect the *mecA* gene that confers methicillin resistance (e.g., in *S. aureus*), the *vanA* and *vanB* genes that confer vancomycin resistance (e.g., in *Enterococcus*), and genes in gram-negative organisms that confer resistance to certain beta-lactam and carbapenem antibiotics (e.g., CTX-M, KPC, NDM). This allows rapid determination of antimicrobial resistance and quicker implementation of appropriate therapy.

POINTS TO REMEMBER

- The skin, skin structures, and normal microbiota play a significant role in protecting the host against microbial invasion and disease.
- The normal skin biota can be involved in the pathogenesis of skin and skin structure infections, particularly if the integrity of the skin is compromised.
- There is an extensive variety of skin and soft tissue infections, which can be classified according to the type of skin lesion produced, the causative organism, or the pathogenesis of the infection (e.g., as a primary entity or secondary to a preexisting infection or systemic manifestation).
- Bacteria, viruses, fungi, and parasites are all important causes of skin and soft tissue infections and diseases.
- *S. aureus* and *S. pyogenes* are common causes of pyoderma.
- Virulence factors of disease-producing organisms (e.g., toxins) can enable the organisms to evade host defense mechanisms, which can result in severe manifestations of infection.
- A compromised immune system can lead to more severe or unusual manifestations of infection and can allow normally innocuous organisms to be pathogenic.
- The occurrence of disease in a host is a function of the underlying host's immunity and virulence of the pathogen.
- Proper specimen collection, correct timing in collection of the clinical specimen, proper laboratory processing of specimens, and clinical context are all important factors to consider when distinguishing between colonization and infection by microorganisms and in making an accurate diagnosis.

LEARNING ASSESSMENT QUESTIONS

1. A hospitalized patient receiving total parenteral nutrition develops candidemia. How is the skin involved in the pathogenesis of this infection?
2. How do folliculitis, furuncles, and carbuncles differ in their pathophysiology and appearance?
3. What are the typical organisms found in diabetic foot infections and gangrene?
4. What is a zoonotic disease, and what are some of the zoonoses that cause skin and soft tissue infection?
5. A patient who has recently received chemotherapy and radiation therapy for a bone marrow transplant develops necrotic skin lesions. A culture from a biopsy of the lesion grows a mold. Which fungi may cause these lesions?
6. Which of the common childhood viral infections are lifelong and can manifest themselves in adults after a period of latency?
7. What are the causes of swimmer's itch, creeping eruption, and ground itch?
8. Which bacteria produce cutaneous manifestations because of toxin production?
9. Which organisms are characterized by the formation of granules and branching filaments in wound exudates?
10. What are some important principles of the laboratory diagnosis of skin and soft tissue infection?
11. A 55-year-old male patient acquired a severe soft tissue infection while working on his boat. The patient has a history of alcohol abuse and hepatitis. Exudates are sent to the microbiology laboratory for Gram stain smear and culture. The culture grows oxidase-positive, fermenting, curved, gram-negative bacilli on sheep blood agar but requires NaCl for growth. Which organism would you suspect?
 - a. *Vibrio vulnificus*
 - b. *Aeromonas hydrophila*
 - c. *Clostridium perfringens*
 - d. *Streptococcus pyogenes*
12. Which of the following conditions is an abscess that extends more deeply into the subcutaneous tissue and may have multiple draining sites?
 - a. Furuncle
 - b. Carbuncle
 - c. Necrotizing fasciitis
 - d. Folliculitis
13. Which condition causes the nail margin to become painful, red, warm, and swollen with pus?
 - a. Furuncle
 - b. Carbuncle
 - c. Eschar
 - d. Paronychia
14. A 5-year-old male patient presents at the family health center with multiple areas of superficial skin loss resembling scalded skin. Cultures from the area show no growth, but nasal cultures yield colonies of catalase-positive, gram-positive cocci. Which organism would you suspect?
 - a. *Streptococcus pyogenes*
 - b. *Streptococcus agalactiae*
 - c. *Staphylococcus aureus*
 - d. *Pseudomonas aeruginosa*
15. A patient presents with an infection that developed after skinning his kill on a deer hunting trip. The patient had minute cuts on the hands that likely acted as ports of entry for organisms. Which condition would you suspect?
 - a. Tularemia
 - b. Plague
 - c. Anthrax
 - d. Melioidosis
16. Catalase negative, beta-hemolytic colonies are isolated from a wound culture taken from a 2-year-old female patient who presented with fluid-filled vesicles on her face. The lesions are characterized as painful, and the areas of inflammation are indurated with raised borders that are sharply demarcated from the adjacent normal skin. Which condition would you suspect?
 - a. Scarlet fever
 - b. Bullous impetigo
 - c. Erysipelas
 - d. Erysipeloid
17. A rancher shows some characteristic lesions on his face described as black scabs. The organisms recovered from culture are gram-positive large rods that are aerobic and produce non-hemolytic, non-motile colonies. Which organism would you suspect?
 - a. *Bacillus anthracis*
 - b. *Nocardia asteroides*
 - c. *Actinomyces israelii*
 - d. *Francisella tularensis*
18. An aspirate from a lesion is taken from a butcher who saw his physician 4 days after infecting his finger while trimming pork products. The infected lesion is sharply defined, painful, and swollen with a purplish red zone. The Gram-stained smear from the culture shows gram-positive small rods. Based on this information, which condition would you suspect?
 - a. Tularemia
 - b. Plague
 - c. Anthrax
 - d. Erysipeloid
19. Which organism is a common cause of eumycetoma?
 - a. *Actinomyces* sp.
 - b. *Madura* sp.
 - c. *Streptomyces* sp.
 - d. *Aspergillus* sp.
20. Which disease results from reactivation of the varicella-zoster virus that presents as vesicular eruption in a unilateral dermatomal distribution?
 - a. Shingles
 - b. Chickenpox
 - c. Rubella
 - d. Measles

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Gastrointestinal infections and food poisoning

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CHAPTER OUTLINE

Anatomic considerations, 870

Approach to diagnosis of the patient with diarrhea, 871

History, 871

Physical examination, 873

Laboratory studies, 873

Clinical presentation and pathogenic mechanisms of acute diarrhea, 873

Enterotoxin-mediated diarrhea, 873

Diarrhea mediated by invasion of the bowel mucosal surface, 874

Diarrhea mediated by invasion of full-bowel thickness with lymphatic spread, 874

Common viral, bacterial, and parasitic pathogens 874

Viral pathogens, 875

Bacterial pathogens, 875

Parasitic pathogens, 879

Diarrhea in special circumstances, 881

Toxic agents of food poisoning, 881

Returning travelers, 881

Immunocompromised hosts, 881

Laboratory diagnosis of gastrointestinal pathogens, 883

Specimen collection and handling, 883

Direct microscopic examination, 883

Culture, 884

Treatment and prevention of diarrhea, 886

Bibliography, 888

4. Determine the factors that place individuals at risk of gastrointestinal infection.
5. Justify the importance of obtaining a medical and travel history in patients presenting with gastrointestinal tract signs and symptoms.
6. Describe the various diarrheagenic *Escherichia coli* strains including their virulence mechanisms.
7. Compare infectious diarrhea in the immunocompromised patient with that in an immunocompetent patient.
8. Identify the common causes of traveler's diarrhea.
9. For each of the bacterial agents described, correlate the predicted results of direct microscopy of the stool specimen, and recommend the selective media for the maximal recovery of the pathogen.
10. Differentiate the clinical manifestations in diarrheal illnesses caused by chemical toxic agents in food poisoning from those mediated by enteric pathogens.
11. Describe modes of treatment and preventive measures of diarrheal events.

KEY TERMS

Achlorhydria

Cholera

Ciguatera

Enterotoxin-mediated diarrhea

Enteric fever

Fecal leukocytes

Median infectious dose (ID₅₀)

Opportunistic pathogens

Orthostatic changes

Scombroid

Traveler's diarrhea

Toxic megacolon

Case in point

A 32-year-old healthy male patient from the United States was visiting his family in a small village in Mexico. The patient consumed some of the local food and drank the water. Four days after arriving in Mexico, he experienced a sudden onset of diarrhea. The diarrhea occurred more than 10 times the first day and was accompanied by nausea, with several episodes of loose, watery stool. The stool contained no gross blood, pus, or mucus. The patient was afebrile but complained of crampy abdominal pain with watery diarrhea and dizziness when standing. His heart rate was rapid, 120 beats/minute (reference range 60 to 100 beats/minute).

OBJECTIVES

After reading and studying this chapter, you should be able to:

1. Explain the normal host defenses at each level of the gastrointestinal tract in preventing infection.
2. Describe the major mechanisms whereby bacteria can cause diarrhea.
3. Associate the onset of symptoms, food ingested, travel history, and clinical manifestations with the possible cause of diarrheal illness.

Issues to consider

After reading the patient's case history, consider:

- The travel, food and water intake, and medical history of the patient
- Clinical symptoms at the time of presentation, and the duration and time of onset of symptoms
- Possible sources of infection

Acute diarrheal illness is one of the most common problems evaluated by health care providers. Colorful names such as *Montezuma's revenge*, *Delhi belly*, *Greek gallop*, *Rome runs*, *Aztec two-step*, and *back door sprint* are evidence of the universal prevalence of acute diarrheal illness across the globe. Acute diarrhea has a significant burden on morbidity and mortality worldwide. In 2018, the Centers for Disease Control and Prevention (CDC) estimated that 48 million people become ill, 128,000 are hospitalized, and 3000 die from foodborne diseases each year in the United States.

Worldwide, diarrhea is responsible for the deaths of 2195 children per day. This is about 1 in 9 child deaths worldwide, making diarrhea the second leading cause of death among children younger than 5 years of age. This is more than human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS), malaria, and measles combined.

Although most healthy people experience a self-limiting illness lasting only a few days, others can experience chronic symptoms, bacteremia, dehydration, and serious sequelae such as malnutrition, severe dehydration, and death. Identifying those individuals who require early treatment is key to limiting morbidity and death. This chapter reviews host and pathogen factors that lead to illness; presents a clinical and laboratory approach to making the diagnosis; discusses common bacterial, viral, and parasitic pathogens; and summarizes treatment and prevention strategies.

Case check 34.1

There are many epidemiologic clues that can provide information regarding the cause of this patient's diarrhea, including his recent travel history to Mexico, dietary history, and health status. The patient is not immunocompromised, there is no evidence of inflammation, and he does not have fever or blood, pus, or mucus in the stool.

The differential diagnosis of acute diarrheal illness is among the broadest in medicine. The chronicity of disease may help narrow the differential diagnosis. A variety of viral, bacterial, and parasitic pathogens may be involved. Toxin-mediated disease may be the cause of diarrhea, and numerous noninfectious causes exist, such as laxative use, tumors, malabsorption, inflammatory bowel disease, and problems with motility caused by hyperthyroidism, irritable bowel syndrome, or surgical reduction of the gut. Evaluating all patients for all possible causes of diarrhea would be prohibitively expensive, so clinical clues must play a role in guiding the most efficient diagnostic testing. The health care provider should determine the most appropriate workup to limit morbidity and death for the patient and prevent transmission of the infection to others.

Anatomic considerations

Although there are exceptions, diarrheal pathogens are usually acquired by ingesting a contaminated food or beverage. Fig. 34.1 shows a diagram of the GI tract. There are host defenses against infection at many levels. Most pathogens that are ingested never reach the intestinal tract because of the acidic environment of the stomach. Normal gastric pH is 1.6 and kills more than 99.9% of coliform bacteria within 30 minutes. Some pathogens, however, are resistant to gastric acids, most notably the cyst phase of parasites and bacterial spores.

The small intestine has a different mechanism to prevent infection. It is constantly in motion (peristalsis). Organisms that rely on adhering to the intestinal wall to cause infection may be hampered by the peristaltic movements. The small intestine and colon have lymphoid tissue that produces antibody, primarily immunoglobulin A (IgA), which may have some effect against pathogenic organisms. The host's normal gut biota is large, with an estimated 10^6 organisms of normal biota per gram of fecal material. Most of these bacteria are anaerobic and outnumber the facultative aerobic bacteria by a factor of

Table 34.1 Microbiota found in the large intestine

Bacterial species ^a	Incidence (%)
Strict anaerobes	
Gram-negative	
<i>Bacteroides fragilis</i>	100
<i>Bacteroides</i> spp.	100
<i>Fusobacterium</i> spp.	100
<i>Prevotella copri</i>	Common
Gram-positive	
Lactobacilli	20–60
<i>Clostridium perfringens</i>	25–35
<i>Clostridium</i> spp.	1–35
<i>Peptostreptococcus</i> spp.	Common
<i>Peptococcus</i> spp.	Common
Facultative anaerobes	
Gram-positive cocci	
<i>Staphylococcus aureus</i>	30–50
<i>Enterococcus</i> spp.	100
β-Hemolytic streptococci, groups B, C, F, and G	0–16
Gram-negative bacilli (Enterobacterales)	
<i>Escherichia coli</i>	100
<i>Klebsiella</i> spp.	40–80
<i>Enterobacter</i> spp.	5–55
<i>Proteus</i> spp.	3–11
<i>Pseudomonas aeruginosa</i>	3–11
Yeast	
<i>Candida albicans</i>	15–30

^aStrict anaerobes are present in ratio of 1000:1 with facultative aerobes.

Modified from Sommers, H. M. (1980). The indigenous microbiota of the human host. In G. P. Youmans, et al. (Eds.), *The biologic and clinical basis of infectious diseases* (2nd ed., p. 83). Philadelphia: Saunders.

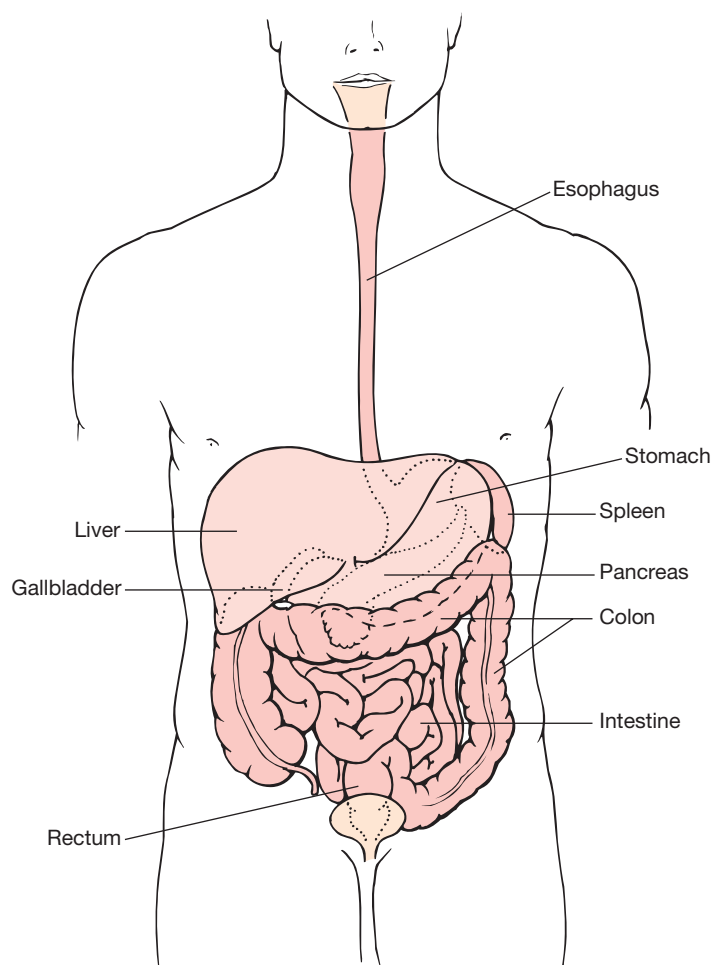


Fig. 34.1 Anatomy of the gastrointestinal tract.

1000:1. Table 34.1 lists the microorganisms frequently found in the large intestine. These established organisms compete with potential pathogens for nutrients and for places to attach to the colon wall. The normal host biota can also produce substances toxic to potential pathogens (e.g., bacteriocins).

Several factors determine the risk of being infected with a GI pathogen. The first is the **median infectious dose (ID_{50})**, the number of organisms that must be ingested to cause a diarrheal illness in 50% of exposed individuals. Even with the best defenses, if an overwhelming number of organisms are ingested, disease can occur. The second factor relates to the host defenses discussed earlier. Patients with inadequate stomach acidity (**achlorhydria**, the absence of hydrochloric acid in gastric secretions), whether primary or because of medications (e.g., proton pump inhibitors that can dramatically alter gastric pH), are more likely than individuals with normal gastric acidity to become ill. Antimicrobial exposure alters the normal biota of the colon and predisposes one to infection with enteric pathogens.

Approach to diagnosis of the patient with diarrhea

The cause of diarrhea can usually be determined by the history, physical examination, and laboratory analysis of a stool

specimen. Table 34.2 summarizes some of the common pathogens involved in diarrhea and the relative frequency of fever, nausea and vomiting, bloody diarrhea, and fecal evidence of inflammation.

History

Diarrhea is defined as an “alteration in a normal bowel movement characterized by an increase in the water content, volume, or frequency of stools.” Most epidemiologic investigations use a definition of more than three bowel movements per day as a definition of diarrhea. The first step in evaluating an individual presenting with an acute diarrheal illness is to take a detailed history and perform a thorough physical examination. Most infections are acquired by ingesting the microorganism. Thus a carefully taken travel and dietary history can point to a causative diagnosis and direct the microbiologic workup.

Group exposures help identify outbreaks in the community, and the history should document if other individuals in contact with the patient have also become ill. Recent travel history is important because travelers to underdeveloped areas are at risk for different types of infections. Recreational activities, such as hiking or backpacking, and even swimming in public pools, have been associated with outbreaks of infectious diarrhea. Seasonality, daycare attendance, and living conditions are also epidemiologic clues to an infectious cause

Table 34.2 Common pathogens involved in diarrhea

Pathogen	Fever	Nausea, vomiting	Bloody stool	Fecal inflammation
<i>Campylobacter</i> spp.	Common	Occurs	Occurs	Common
<i>Salmonella</i> spp.	Common	Occurs	Occurs	Common
<i>Shigella</i> spp.	Common	Common	Occurs	Common
Enterohemorrhagic <i>Escherichia coli</i>	Atypical	Occurs	Common	Often not found
<i>Clostridioides difficile</i>	Occurs	Not characteristic	Occurs	Common
<i>Yersinia enterocolitica</i>	Common	Occurs	Occurs	Occurs
<i>Entamoeba histolytica</i>	Occurs	Variable	Variable	Variable
<i>Cryptosporidium</i> spp.	Variable	Occurs	Not characteristic	None to mild
<i>Cyclospora</i>	Variable	Occurs	Not characteristic	Not characteristic
<i>Giardia duodenalis</i>	Not characteristic	Occurs	Not characteristic	Not characteristic
Viruses	Variable	Common	Not characteristic	Not characteristic

Modified from Thielman N. M., & Guerrant, R. L. (2004). Clinical practice. Acute infectious diarrhea. *The New England Journal of Medicine*, 350, 38.

of diarrhea. All of these historic factors must be considered when evaluating a patient with gastrointestinal (GI) disease.

Other key questions that can be asked include the following:

- What is the duration of symptoms? Acute diarrhea is generally defined as symptoms lasting fewer than 14 days. If symptoms last longer than 14 days, the term *persistent diarrhea* is sometimes used. When diarrhea lasts longer than 30 days, the term *chronic diarrhea* is applied. These categories can help narrow the differential diagnosis because many causes of diarrhea are self-limiting.
- Are there associated symptoms of inflammation? Fever, bloody stools, and tenesmus can be symptoms of a more invasive process.
- Does the patient have a history of previous GI symptoms? A positive history may suggest other illnesses, such as inflammatory bowel disease or irritable bowel syndrome, rather than an infectious cause.
- Does the patient have an underlying illness? For example, patients with AIDS or who are immunosuppressed because of chemotherapy or organ transplantation are at risk for organisms not routinely considered pathogens in otherwise healthy individuals.
- Is the patient taking any medications? Some medications (e.g., several common antidepressant medications, medications for HIV infection) are known to cause GI side effects. A recent history of antimicrobial use may also suggest an infection with *Clostridioides difficile*.

Travel to countries with less effective sewage facilities increases the risk of acquiring an enteric pathogen. **Traveler's diarrhea** is usually caused by enterotoxigenic *Escherichia coli* (ETEC). This disease has a short incubation period (usually between 5 and 15 days after arrival) and normally lasts from 1 to 5 days. If a diarrheal illness develops weeks after a traveler returns home, a parasitic infection such as *Giardia duodenalis* (synonyms *G. lamblia* and *G. intestinalis*) or *Entamoeba histolytica* infection would be more likely.

Because most diarrheal pathogens are acquired by ingesting a contaminated food or beverage, a detailed food history going back 3 days before the onset of symptoms is helpful. Water, unpasteurized milk, poultry, beef, and shellfish are

Table 34.3 Common food vehicles for specific pathogens or toxins

Vehicle	Pathogen or toxin
Undercooked chicken	<i>Salmonella</i> spp., <i>Campylobacter</i> spp.
Eggs	<i>Salmonella</i> spp. (especially <i>S. Enteritidis</i>)
Unpasteurized milk	<i>Salmonella</i> , <i>Campylobacter</i> spp., <i>Yersinia</i> spp.
Water	<i>Giardia duodenalis</i> , noroviruses, <i>Campylobacter</i> spp., <i>Cryptosporidium</i> spp., <i>Cyclospora</i>
Fried rice	<i>Bacillus cereus</i>
Fish	
Shellfish	<i>Vibrio cholerae</i> , <i>V. parahaemolyticus</i> , <i>V. vulnificus</i> , other <i>Vibrio</i> spp., neurotoxic shellfish poisoning, paralytic shellfish poisoning, noroviruses, and sapoviruses
Tuna, mackerel, mahi-mahi	Scombroid poisoning
Grouper, amberjack, snapper	Ciguatera
Sushi	<i>Anisakis</i> spp.
Beef, gravy	<i>Salmonella</i> spp., <i>Campylobacter</i> spp., <i>Clostridium perfringens</i>

Modified from Goodman, L. J. (1993). Diagnosis, management, and prevention of diarrheal diseases. *Current Opinion in Infectious Diseases*, 6, 88.

often responsible for causing foodborne infections. **Table 34.3** lists foods that are commonly linked to infectious diarrhea and the organisms that are typically involved. The duration of illness can also be helpful in narrowing the differential diagnosis. Patients with an invasive bacterial pathogen and bloody stools usually present earlier than patients with *Giardia* or other parasitic infections. Some patients with toxin-mediated illness may present very early in the course of symptoms or never present to a clinician because of the relatively brief course of these illnesses. If the patient is currently taking or has recently received antimicrobial agents, *C. difficile* would be an important consideration.

The patient should be asked about other medical conditions because diarrhea can be caused by noninfectious diseases, such as inflammatory bowel disease, malabsorption, or radiation therapy. If the patient is immunosuppressed from AIDS, chemotherapy, or organ transplantation, or because of other medical reasons, opportunistic pathogens should be considered. Diarrhea can also be a side effect of some medications.

Physical examination

The first step in the physical examination of a patient with a diarrheal illness is to determine the patient's state of hydration. There are several signs suggestive of dehydration. For example, patients may have a sunken appearance to the eyes, dry oral membranes, or loss of skin resiliency, known as *skin tenting*. This is tested by gently pinching the skin on the back of the hand or over the sternum. In a dehydrated patient, the skin remains in a pinched or tented position. Patients may also have a decrease in blood pressure or an increase in heart rate on moving from a supine to a seated or standing position (**orthostatic changes**). If the dehydration is severe enough, the patient may have changes in mental status or dysfunction of other organ systems (e.g., kidney failure). If the patient has a fever, it may be a clue that the patient is infected with an invasive pathogen.

Examination of the abdomen may also help lead to a diagnosis. Frequently, the abdomen is diffusely tender. Examination with a stethoscope reveals that bowel sounds are present, sometimes hyperactive in quality. If the pain is localized to only one section of the abdomen, there is severe pain on palpation of the abdomen, or bowel sounds are absent, the patient should be evaluated for complications of diarrheal infection (e.g., **toxic megacolon** or intestinal perforation) or a different disease process, such as appendicitis, pancreatitis, or ovarian torsion.

Laboratory studies

Evaluation of the patient's peripheral blood cell count may reveal a leukocytosis in invasive infections. Anemia may be present in cases of severe GI blood loss or hemolytic infection. Thrombocytopenia (low platelet count) may be present in some infections. Evaluation of the patient's blood chemistry test results can show electrolyte abnormalities. Examination of the stool for red blood cells (RBCs) and evidence of inflammation (**fecal leukocytes** in the stool; Fig. 34.2) or testing for

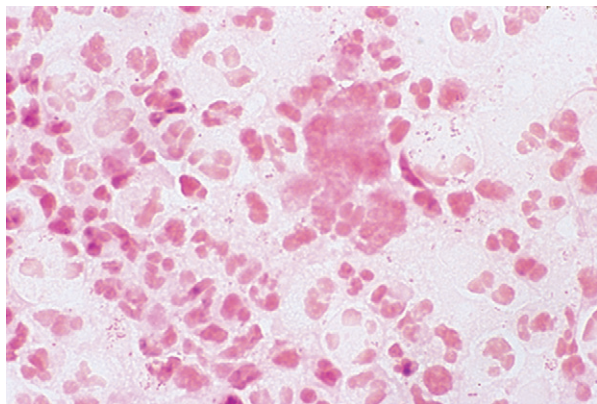


Fig. 34.2 Gram stain of a direct fecal smear showing the presence of white blood cells, indicative of an invasive process and not an enterotoxin.

fecal lactoferrin (a neutrophil marker associated with inflammation) may help differentiate those patients who have invasive disease from those patients with toxin-mediated illnesses, viral illnesses, or parasitic infections.

Case check 34.2

A stool sample was submitted for testing. It was negative for white blood cells (WBCs) and lactoferrin, which is consistent with the history of lack of fever and blood in his stools. Combined with the clinical history (short duration of symptoms and travel history), the most likely cause is a toxin-mediated process such as that associated with enterotoxigenic *E. coli* (ETEC), the organism that causes approximately 50% of traveler's diarrhea. Viral pathogens can also cause a noninflammatory diarrhea but are less likely in this case.

Clinical presentation and pathogenic mechanisms of acute diarrhea

Based on the history, physical examination, and preliminary laboratory findings, the health care provider should be able to shorten the list of potential pathogens. The workup of the patient can then be completed in conjunction with the microbiologist to determine appropriate diagnostic methods. Several diagnostic methods exist to determine the cause of acute diarrhea. Carefully taking the history, as discussed earlier, can help formulate a differential diagnosis of expected pathogens. Another classic approach is to categorize acute diarrhea by pathogenic mechanisms. Based on certain signs and symptoms and stool sampling, diarrhea can be characterized as enterotoxin mediated or invasive. Although there may be significant overlap between these groups, this categorization is useful as an initial diagnostic approach.

Enterotoxin-mediated diarrhea

Case study

A 25-year-old female patient recently attended a picnic at a family reunion. A few hours after returning home, she experienced a sudden onset of vomiting and frequent diarrhea, with abdominal cramping. She reported not having any fever. She felt lightheaded when standing and had a rapid heart rate, 130 beats/minute (reference range 60 to 100 beats/minute). The stool was watery, without any pus or gross blood. Her symptoms resolved the next day. It was determined that several other family members who had attended the reunion developed similar symptoms.

The lack of fever and absence of blood or pus in the stool suggests an enterotoxin-mediated illness. Bacteria associated with enterotoxin production do not invade the gut wall, and the toxin does not elicit an inflammatory response. Because there is no systemic inflammatory response, patients usually do not have a fever. In the Case Study, the patient's symptomatic lightheadedness and rapid heart rate suggest significant volume depletion. Initial treatment is aimed at rehydrating the patient by administering oral or intravenous (IV) fluids.

The rapid onset of symptoms following food ingestion suggests an enterotoxin-mediated cause. In these cases, the preformed toxin is present in the ingested food. Because toxin can act proximally in the bowel (the small intestine), the incubation period is relatively short, usually less than 12 hours. When patients present to a health care provider, they usually report an illness that started the day of or the day before presentation. They typically have several (sometimes >20/day) nonbloody, watery stools and frequently have vomiting and abdominal cramping, particularly during defecation. Stool examination will be negative for RBCs and WBCs.

There are several causative organisms for **enterotoxin-mediated diarrhea**. ETEC accounts for the largest percentage of cases of diarrhea in travelers to underdeveloped areas. Other organisms responsible for enterotoxin-mediated disease include *Vibrio cholerae*, *Staphylococcus aureus*, *Clostridium perfringens*, and *Bacillus cereus*. When *S. aureus* is implicated, the source of infection can often be traced to food handlers with small, localized abscesses in the nail beds known as *paronychias*. Similar clinical syndromes are caused by other bacterial pathogens (e.g., *Plesiomonas* or *Aeromonas*) or by viruses. Noninvasive parasitic infections such as giardiasis and cryptosporidiosis or infection with *Cystoisospora belli* can also produce an afebrile diarrhea, with a negative microscopic stool examination result. However, patients with these infections typically have fewer stools per day than those with enterotoxin-mediated disease, and they usually have a more prolonged illness.

Case check 34.3

The history of food ingestion and rapid onset of symptoms in this case indicate a toxin-mediated process such as that caused by *S. aureus* or *B. cereus*. However, it is important to remember that viral and noninvasive parasitic infections such as giardiasis and cryptosporidiosis can also result in normal stool examination findings in patients with diarrhea.

Diarrhea mediated by invasion of the bowel mucosal surface

Case study

A 56-year-old male patient was being treated with clindamycin for a skin infection. After 4 days of therapy, he developed a fever and had about 15 episodes of diarrhea per day. A peripheral blood count revealed a new leukocytosis. Stool examination revealed fecal WBCs and RBCs as well as the presence of *C. difficile* toxin. The patient was treated with orally administered metronidazole, with resolution of symptoms.

The most common organisms in this category are *Salmonella enterica*, *Campylobacter* spp., *Shigella* spp., some *E. coli* strains, and *Entamoeba histolytica*. In contrast with enterotoxin-mediated diarrhea, organisms that invade the bowel mucosa cause an inflammatory response, characterized by the presence of fecal leukocytes, fever, and leukocytosis. Spread of the infection to the regional lymph nodes is unusual. However, *Salmonella* Typhi does invade the tissue spreading to the blood stream causing typhoid fever. Because the organisms

must first replicate in the colon and then invade the mucosal surface, the incubation period is usually longer than that of enterotoxin-mediated diarrhea. Because these infections involve only the superficial mucosal surface of the bowel, bacteremia or metastatic infection is infrequent. Patients may present with true dysentery syndrome, characterized by gross blood and pus in the stool. Other pathogens, such as enterohemorrhagic *E. coli*, cause bloody stools, but fever and fecal leukocytes are less common findings. *E. histolytica* has a longer incubation period than other enteric pathogens.

Case check 34.4

This patient has evidence of invasive disease based on his fever and the presence of fecal WBCs and RBCs. *C. difficile* is a common cause of invasive diarrhea after antimicrobial therapy.

Diarrhea mediated by invasion of full-bowel thickness with lymphatic spread

Case study

A 30-year-old female patient returning from South America presented to the emergency department with fever. Laboratory studies showed a mild anemia, low platelet count, and mild neutropenia. Blood cultures were sent to the laboratory. One week later, the patient developed a watery diarrhea, and stool cultures were sent to the laboratory. Stool and blood cultures were positive for *Salmonella* Typhi. The patient was treated with ciprofloxacin, with reduction of symptoms after several days.

The most common invasive enteric organisms are *Salmonella* Typhi and *Yersinia enterocolitica*. The incubation period for these infections is about 1 to 3 days. These organisms can invade the bowel wall, causing bacteremia and mesenteric lymphadenitis that can be mistaken for appendicitis. Diarrhea may be absent at the onset of the disease. RBCs and WBCs are often present in the stool. Gross blood in the stool occurs in about 25% of patients with *Y. enterocolitica* infection. Patients with *Salmonella* Typhi infection may become chronic carriers and unknowingly spread the infection to others.

Case check 34.5

This patient improved with ciprofloxacin therapy, but there is growing resistance among *S. Typhi* isolates to fluoroquinolones, especially in Asia, where azithromycin or ceftriaxone is now the recommended empiric regimen. Complications of untreated severe typhoid fever can include severe intestinal hemorrhage and intestinal perforation.

Common viral, bacterial, and parasitic pathogens

This section discusses some of the most important viral, bacterial, and parasitic causes of infectious diarrhea.

Viral pathogens

Case study

A 2-year-old male patient who attends daycare developed vomiting and diarrhea. He had a low-grade fever taken orally of 100.0°F (37.8°C), reference range 97.6°F (36.4°C) to 99.3°F (37.4°C). When the parents questioned the daycare employees, they learned that several other daycare children had similar symptoms, and several children were absent from the daycare because of illness. Examination of stool from the patient did not reveal any fecal leukocytes. The child was encouraged to drink copious fluids, and his symptoms resolved after a few days.

Viruses were suspected to be a cause of gastroenteritis from the 1940s, but no pathogen was identified in feces of sick individuals until 1972. Since then, the number of identified viral pathogens has steadily increased. Viruses pose a challenge to the microbiologist because of their small size, which precludes the use of light microscopy. Techniques used for their identification include antibody testing, antigen detection, and nucleic acid amplification of the viral genome. Due to cost and technical demands, culture in cell lines and electron microscopy are no longer used routinely.

Rotaviruses

Members of the family Reoviridae, **rotaviruses** are nonenveloped and have a 70-nm icosahedral structure. They are classified into groups, subgroups, and serotypes based on the capsid protein antigens. Rotaviruses are the most common cause of diarrhea in children younger than 5 years of age, causing an estimated 130 million episodes of diarrhea worldwide each year. Though this pathogen is primarily associated with children, it is also relevant for adults and can cause severe and protracted diarrhea in immunocompromised hosts. Rotavirus infects cells of the villi of the small intestine, leading to epithelial atrophy and proliferation of cells with secretory capacity. These changes may decrease the absorptive capacity of the bowel and increase the amount of intestinal water and electrolytes in the lumen, which results in diarrhea. Treatment is mainly supportive care. Prevention is now possible because a vaccine against rotavirus is available, and it helps reduce morbidity- and death-related diarrheal illness.

Enteric adenoviruses

Adenoviruses, members of the family Adenoviridae, are nonenveloped 70-nm viruses with icosahedral symmetry. Adenoviruses have been implicated in many illnesses, ranging from the common cold and epidemic keratoconjunctivitis (pink eye) to infectious diarrhea. It is not uncommon to have a preceding upper respiratory tract infection followed by infectious diarrhea with this agent. There are multiple serotypes, and it is postulated that associations between serotypes and syndromes can differ by geographic region. Two adenovirus serotypes, 40 and 41, are most frequently associated with infectious diarrhea. Diarrhea results from a mechanism similar to that for rotaviruses. This virus causes 1% to 8% of cases of diarrhea in the developed world.

Caliciviruses

Members of the family Caliciviridae, the caliciviruses are characterized by 32 cup-shaped depressions along the surface. Two genera are associated with GI disease in humans, *Norovirus* (formerly *Norwalk-like viruses*) and *Sapovirus* (formerly *Sapporo-like viruses*). The noroviruses are named after the original strain, Norwalk virus, which was first isolated in 1968 in patients from a town with that name in Ohio. These viruses cause an acute, self-limiting diarrheal illness associated with vomiting and low-grade fever. The disease is highly contagious and is transmitted by the fecal-oral route via contaminated food or water, by environmental fomites, or from person to person. The virus can be transmitted by the aerosolization of vomitus. Outbreaks have been reported aboard cruise ships.

Astroviruses

Members of the family Astroviridae, these viruses express different morphologies depending on the pH of the media to which they are exposed. The original characterization as a small, 28-nm virus that appears as a five- or six-pointed star is the morphology at alkaline pH; the virus has a 41-nm icosahedral appearance with defined spikes at other pH values. The mechanism of the diarrhea resulting from these viruses is not well understood, but animal studies suggest that an osmotic diarrhea may develop from inflammatory infiltrates in the lamina propria and the intestinal villus atrophy. These agents usually cause infection in older adults or the very young.

Coronaviruses

Diarrhea may be present as systemic manifestations of other viral infections. Notably, severe acute respiratory syndrome–coronavirus-2 (SARS-CoV-2) and its clinical disease, Coronavirus Disease 2019 (COVID-19), may present initially with malaise and diarrhea. Though this condition is primarily respiratory, atypical presentations can occur with GI manifestations as main symptoms. This can occur with other viral respiratory illnesses as well. Understanding the local prevalence of SARS-CoV-2 in a community and assessing whether the patient has sick contacts may help increase suspicion for COVID-19. Other members of the family Coronaviridae have also been associated with gastroenteritis.

Case check 34.6

Viruses such as the rotavirus, adenovirus, and norovirus usually cause a self-limiting, noninflammatory diarrhea. Treatment is supportive.

Bacterial pathogens

Case study

A 30-year-old male patient hosted a barbecue for a football weekend at which he served chicken. On the Tuesday after the event, the patient and several other people who had attended the barbecue developed fever and diarrhea. Stool studies from the patient revealed RBCs, WBCs, and numerous curved, gram-negative rods. After several days, the patient and other attendees recovered without complications.

Bacteria are a common cause of infectious diarrhea. Several agents are known to cause diarrhea by various mechanisms, ranging from toxin production (performed in food or produced in the intestine) to mucosal invasion. Antimicrobial agents are used for the treatment of some of these illnesses. The clinical microbiologist has an important role in identifying these organisms.

Campylobacter jejuni

Campylobacter jejuni is the most common bacterial cause of gastroenteritis in the world. For most cases, antimicrobial therapy is not necessary. Because of the widespread misuse of antimicrobial agents, these organisms are developing significant resistance to agents commonly used to treat diarrheal illnesses (e.g., fluoroquinolones). Guillain-Barré syndrome, a life-threatening neurologic disease, and reactive arthritis are complications of infection by *C. jejuni*.

Infection with this organism usually results in a self-limiting disease characterized by fever, abdominal cramping, and diarrhea. The incubation period is 2 to 5 days, but in some cases extends up to 10 days. Diarrhea is often preceded by a period of fever, malaise, myalgias, and abdominal pain. Stools may have gross blood and pus present. The diarrhea usually lasts 2 to 3 days, but abdominal discomfort may persist for several days after resolution of the diarrhea. The pathogen can be cultured from stool for several weeks after the illness has subsided. The environmental reservoirs of *Campylobacter* are wild and domestic animals, typically birds.

Contaminated poultry is often the source of the infection. Other outbreaks have been traced to contaminated meat, water, and unpasteurized milk. Although there is prolonged fecal shedding of the organism, there is little person-to-person transmission of the infection. If antimicrobials will be used to treat the infection (in the case of immunocompromised patients or those with severe or prolonged illness), a macrolide antibiotic is preferred because of the increasing rates of fluoroquinolone resistance. Because the organism is heat sensitive, thorough cooking of meat and poultry is effective in preventing human infection. Although food irradiation may be effective in decreasing the amount of bacteria present in uncooked food, this technique is feared by the public. *Campylobacter coli* is also a relatively common cause of gastrointestinal tract infections and produces signs and symptoms similar to *C. jejuni*.

Salmonella species

Gastroenteritis and food poisoning

Nontyphoidal *Salmonella* spp. can lead to gastroenteritis through the ingestion of contaminated meat, poultry, eggs, dairy products, and aquaculture-farmed fish and shellfish. A wide variety of nontyphoidal *Salmonella* serotypes are present in animal hosts, most notably birds and reptiles.

Symptoms usually start between 6 and 48 hours after ingestion of contaminated foods and consist of nausea, vomiting, and diarrhea. Patients may also develop fever, headaches, and myalgias. The illness is usually self-limiting and resolves within a few days. Antimicrobials are not recommended in most cases because they do not shorten the course of the illness and can promote long-term carriage.

Enteric fever

Salmonella Typhi causes the most severe form of **enteric fever**, typhoid fever. Unlike the other serotypes of *Salmonella*,

humans are the only known host for this pathogen. The infection is commonly spread through fecally contaminated food or water. In the United States, there are approximately 350 cases per year. Mortality can be as high as 15%.

In the initial stages of the infection, the pathogen invades the small and large bowel walls, creating an inflammatory response. The bacteria survive inside host cells and are therefore termed *intracellular pathogens*. The infection spreads throughout the body via the regional lymph nodes and bloodstream. The initial symptoms of infection are headache, fever, general malaise, and abdominal tenderness. Once the organism has spread throughout the body, it reaches the gallbladder and Peyer patches in the colon, initiating the diarrheal stage of the illness. The organism can frequently be recovered from blood and later stool cultures. Immunocompromised hosts and patients with HIV have a higher risk of invasive *Salmonella* disease and bacteremia. The incidence of salmonellosis in HIV-positive patients has been estimated to be as high as 20 to 100 times greater than that in an immunocompetent host. Thus an HIV test should be considered for patients with *Salmonella* bacteremia. *Salmonella enterica* serotype Enteritidis and *Salmonella enterica* serotype Typhimurium are the two most important serotypes of *Salmonella* transmitted from animals to humans in most parts of the world. These agents typically produce a gastroenteritis without bacteremia.

Appropriate antimicrobial use results in clinical improvement; however, stool cultures often remain positive, which can serve as a source of infection for other individuals. Some patients can develop chronic colonization of their gallbladder and biliary tree, leading to persistent shedding of the organism, with potential transmission to others (the classic Typhoid Mary case).

Shigella species

Very closely related to *Escherichia* spp., *Shigella* spp. are responsible for a diarrheal illness characterized by gross blood and pus in the stool. There are four recognized *Shigella* spp.: *S. sonnei* (the most common in the United States), *S. flexneri*, *S. dysenteriae*, and *S. boydii*. Because the ID₅₀ of this organism is very low (possibly <100 organisms), it is among the most easily communicable bacterial diarrheal pathogens. Invasion into the intestinal mucosa leads to ulceration and a marked inflammatory response; the organism usually does not invade past the mucosa or spread into the bloodstream. The bacteria produce several toxins, including the Shiga toxin, which can have cytotoxic, enterotoxic, and neurotoxic effects. In 2016, there were 12,597 cases of shigellosis, in the United States. Children 1 to 4 years old had the highest incidence of all age groups. Multiple outbreaks have occurred in recent years, including outbreaks in people experiencing homelessness in the Pacific Northwest in 2021 and other major metropolitan areas.

The incubation period of the disease can range from 1 to 7 days, but most patients develop symptoms within 12 to 50 hours after exposure. The initial symptoms are fever, malaise, fatigue, and anorexia. A watery diarrhea with abdominal cramping and tenesmus then develops, which may progress to the patient having gross blood and pus in the stool. Although the disease is often self-limiting, antimicrobial treatment is recommended as multiple randomized controlled trials have consistently shown clinical benefit with antimicrobial use. Resistance is on the rise and can vary, with rising trimethoprim/sulfamethoxazole resistance. Quinolones are favored when susceptibility is unknown.

Table 34.4 Diarrheagenic *Escherichia coli*

Group	Major virulence factors	Reported food sources
ETEC	Adhesins LT and ST toxins	Fresh fruits and vegetables, scallops, tuna paste, soft cheeses
EIEC	Invasion proteins	Cheese, guacamole
EHEC	Intimin (adherence to intestinal mucosa)*Shiga toxins	Undercooked beef, sausage, chicken, lunch meats, deer jerky, lettuce, radishes, alfalfa sprouts, potatoes, milk, apple juice, cider, cheese curds
EPEC	Intimin (adherence to intestinal mucosa) Type IV bundle-forming pili Surface-associated filaments Translocated intimin receptor ^a	Fresh fruits and vegetables, likely infant formula
EAEC	Aggregative adherence fimbriae Dispersin Plasmid-encoded toxin	Likely foodborne, possibly fruits and vegetables, other food sources uncertain
DAEC	Adhesins	Likely contaminated food or water and also through person-to-person contact

DAEC, Diffusely adherent *E. coli*; EAEC, enteroaggregative *E. coli*; EHEC, enterohemorrhagic *E. coli*; EIEC, enteroinvasive *E. coli*; EPEC, enteropathogenic *E. coli*; ETEC, enterotoxigenic *E. coli*; LT, heat labile; ST, heat stable.

^aIntimin and translocated intimin receptor are encoded by the locus of enterocyte effacement.

Escherichia coli

Although this species is a normal inhabitant of the human GI tract, six different strains of *E. coli* have been associated with disease (Table 34.4). *E. coli* O157 illnesses are most often linked to vegetable row crops (such as leafy greens) and beef.

Enterotoxigenic *Escherichia coli*

Enterotoxigenic *E. coli* (ETEC) produces adhesins that bind to intestinal mucosa and enterotoxins, which are heat stable enterotoxin (ST) or heat labile enterotoxin (LT) and are responsible for diarrhea. LT is similar to *V. cholerae* toxin. ST binds to guanylate cyclase, leading to increased intracellular cyclic guanosine monophosphate (cGMP) levels and active efflux of water and electrolytes from the intestinal cells. Clinically, patients have a watery diarrhea that lacks RBCs and WBCs. This is a common cause of traveler's diarrhea.

Enteroinvasive *Escherichia coli*

Enteroinvasive *E. coli* (EIEC) produces an infection similar to *Shigella* infection. Patients usually develop a watery diarrhea similar to that produced by ETEC. Some patients then progress to an invasive-type diarrheal syndrome, with fever, abdominal cramping, and bloody stools. Fecal leukocytes are abundant.

Enterohemorrhagic *Escherichia coli*

Enterohemorrhagic *E. coli* (EHEC) strains produce Shiga toxin 1 and/or Shiga toxin 2 and possess the locus of enterocyte

effacement that mediates attachment to enterocyte membrane and destruction of the brush border microvilli. Infection usually starts as a watery diarrhea. However, over the next 1 or 2 days, abdominal pain increases, and stools become bloody. Stool may contain some leukocytes, so there may be some confusion between this infection and EIEC infection or shigellosis. Although the disease is usually self-limiting, patients can develop hemolytic-uremic syndrome (HUS), characterized by hemolytic anemia, low platelet count, and kidney failure. The *E. coli* strain O157:H7 is among the principal causes of this syndrome. It is uncertain whether antimicrobial therapy decreases the probability of developing HUS. There is some concern that subtherapeutic antibiotic dosing may actually increase the risk of developing the disease by stimulating bacterial toxin production.

Enteropathogenic *Escherichia coli*

Enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC) are collectively referred to as *enteroadherent E. coli* in some classification schemes. Although the mechanism of diarrhea is not completely understood, it seems that the organisms express various factors that facilitate their adherence to intestinal cells. This causes disruption and destruction of the brush border of the cells and other intestinal cell derangements, reducing the absorptive capacity of the cells and leading to electrolyte abnormalities and diarrhea. These organisms commonly affect children in nurseries and daycare centers. Patients often have low-grade fever, vomiting, and diarrhea.

Enteroaggregative *Escherichia coli*

EAEC also adheres to the intestinal surface but in a more clumped or aggregative fashion. These organisms apparently first adhere to the intestinal wall via various adhesive molecules, cause an increase in the production of mucus, and then induce an inflammatory response by host cytokine release. EAEC is being increasingly recognized as a cause of traveler's diarrhea. Infections with these organisms can range from asymptomatic to a chronic watery diarrhea.

Diffusely adherent *Escherichia coli*

DAEC is characterized by a diffuse adherence pattern on cultured HeLa or HEP-2 cells. These strains express unique adhesion molecules first described in urinary pathogenesis. Later it was shown that these strains were responsible for diarrhea in children 18 months to 5 years of age, but not in adults. In fact, these strains can be part of the asymptomatic intestinal microbiota in older children and adults. Clinical studies have not demonstrated characteristic signs and symptoms in children infected with DAEC. Although a large percentage of children present with diarrhea, abdominal pain, and vomiting, the frequencies of these complaints are not different from those of children infected with EPEC, EAEC, or ETEC.

Vibrio species

Vibrio species are curved, gram-negative rods, similar in appearance to *Campylobacter*. They are usually found in water environments and can contaminate fish and shellfish. Although many species of *Vibrio* are implicated in human disease, one of the best known is *V. cholerae*, the causative agent of **cholera**. This organism creates a well-studied enterotoxin that consists of two subunits (a core A subunit surrounded

by five B subunits). The B subunits permit attachment to the small bowel mucosa; the A subunit then enters the cell. Once in the cell, through various enzymatic effects, the toxin results in an increase in cyclic adenosine monophosphate (cAMP), which causes the cell to secrete large amounts of water and electrolytes. This increased secretion causes the rice water stools characteristic of cholera. Many liters of stool can be produced daily, and patients without adequate medical care are at risk of death from dehydration. Although the quantity of diarrhea can be impressive, it is toxin mediated and noninflammatory, so the stools are free from RBCs and WBCs.

There are many serogroups (serovars) of *V. cholerae*. Serogroups O1 and O139 have been implicated as causes of epidemic cholera. The other serogroups can also cause illness but have not been associated with the epidemic form of the disease. Two other *Vibrio* spp. that can cause GI illness are *Vibrio parahaemolyticus* and *Vibrio vulnificus*, although other species have been implicated as well. Without treatment, case fatality rates can be as high as 50%.

Vibrio parahaemolyticus is the most common *Vibrio* species responsible for disease in the United States and is fairly common worldwide. Patients with this infection, unlike those with classic cholera, can have fever and evidence of fecal inflammation, suggesting that there is some potential for mucosal invasion. *V. vulnificus* is the most virulent of all non-cholera vibrios, causing a fulminant illness characterized by sepsis and bullous skin lesions in patients with a recent ingestion of shellfish (e.g., raw oysters). This *Vibrio* species is part of the marine microbiota, making it a common colonizer of a large percentage of oysters harvested in the warmer months. Compromised patients, particularly those with liver disease, are more susceptible to the development of fulminant disease.

Yersinia enterocolitica

Related to the organism responsible for bubonic plague, *Yersinia enterocolitica*, a gram-negative bacillus, can produce gastroenteritis. Infections with this organism, which are more common in the winter months, have been linked to meat, unpasteurized milk, other dairy products, and chitterlings. Refrigeration does not prevent growth of this organism.

Patients can present with a self-limiting enteritis consisting of fever, nausea, diarrhea, and abdominal pain or can develop a more invasive disease, with spread of the organism to the mesenteric lymph nodes. This mesenteric lymphadenitis can be mistaken for appendicitis. Stool studies will show both RBCs and WBCs. Because the organism has the ability to invade and spread throughout the body, distant foci of infection and abscesses can also develop.

Clostridioides difficile

First identified in 1935 as an anaerobic, gram-positive, spore- and toxin-producing organism, *C. difficile* was later found to be a causative agent of antibiotic-associated diarrhea. *C. difficile*-associated disease (CDAD) was initially reported in patients receiving clindamycin; however, almost all classes of antimicrobials have been linked to this infection. The initial event in the pathogenesis of the disease is the antimicrobial-induced disruption of the indigenous bacterial biota of the colon that allows the overgrowth of *C. difficile*. The organism produces two different exotoxins, toxins A and B, which bind to surface epithelial cell receptors, leading to inflammation, mucosal injury, and

diarrhea. The development of characteristic pseudomembranes in the colonic mucosa, composed of neutrophils, fibrin, mucin, and cellular debris, is pathognomonic of CDAD. In mild cases, these pseudomembranes are absent.

Other important risk factors for asymptomatic colonization and infection with this bacterium include a recent hospitalization and residence in a long-term care facility. Cases of community-acquired disease, unrelated to antimicrobial use, have been described. The organism is transmitted from person to person via the fecal-oral route and can easily be passed from a colonized patient to other patients in the hospital by health care personnel who do not observe proper hand hygiene. *C. difficile* has been cultured from inanimate hospital surfaces.

The clinical presentation of CDAD ranges from a mild watery diarrhea to a life-threatening toxic megacolon, which requires surgical intervention. Patients with severe disease often present with abdominal pain, leukocytosis, and fever in addition to diarrhea. An epidemic and more virulent strain of *C. difficile* has been identified. This epidemic strain, referred to as BI/NAP1/027, has increased virulence that is attributed to the overexpression of toxin-encoding genes producing larger amounts of toxins, which causes a more aggressive form of the disease compared with the nonepidemic strains.

Diagnosis of CDAD is often made on clinical grounds or by detecting the presence of *C. difficile* toxins in a stool sample. The finding of pseudomembranes during rectosigmoidoscopy is highly suggestive of the disease. Orally administered metronidazole or vancomycin remains the mainstay treatment. Vancomycin has been shown to be superior to metronidazole. Recent studies have shown better performance by fidaxomicin, and it is now the recommended first-line agent. However, vancomycin remains an acceptable first choice when fidaxomicin is not available.

Listeria monocytogenes

A gram-positive, non-spore-forming facultative anaerobic bacillus, *L. monocytogenes* is best known for causing systemic disease, such as meningitis. However, several outbreaks of gastroenteritis have implicated this intracellular pathogen. The presence of this organism in an asymptomatic individual is not necessarily a cause for concern because 5% to 10% of healthy adults transiently carry *L. monocytogenes* in the bowel at any given time. Most outbreaks have been linked to contaminated deli meats and cheeses. In 2021, frozen, fully cooked chicken products, such as chicken strips and diced chicken, and products made with fully cooked chicken were associated with *L. monocytogenes* contamination causing several illnesses, hospitalizations, and deaths.

Helicobacter species

Although *H. pylori* (Fig. 34.3) does not cause diarrhea, it has been associated with peptic ulcer disease and gastric carcinoma. Infection with *H. pylori* is common. Active infection can be diagnosed by detecting the organism on a gastric biopsy specimen or through a urea breath test (Fig. 34.4). This testing is based on the organism's high urease activity. ¹³C- or ¹⁴C-labeled urea will, when ingested, produce labeled CO₂ in the patient's breath if the organism is present. Other *Helicobacter* spp. (e.g., *H. cinaedi*, *H. fennelliae*) are presumed to play a role in diarrheal illnesses.

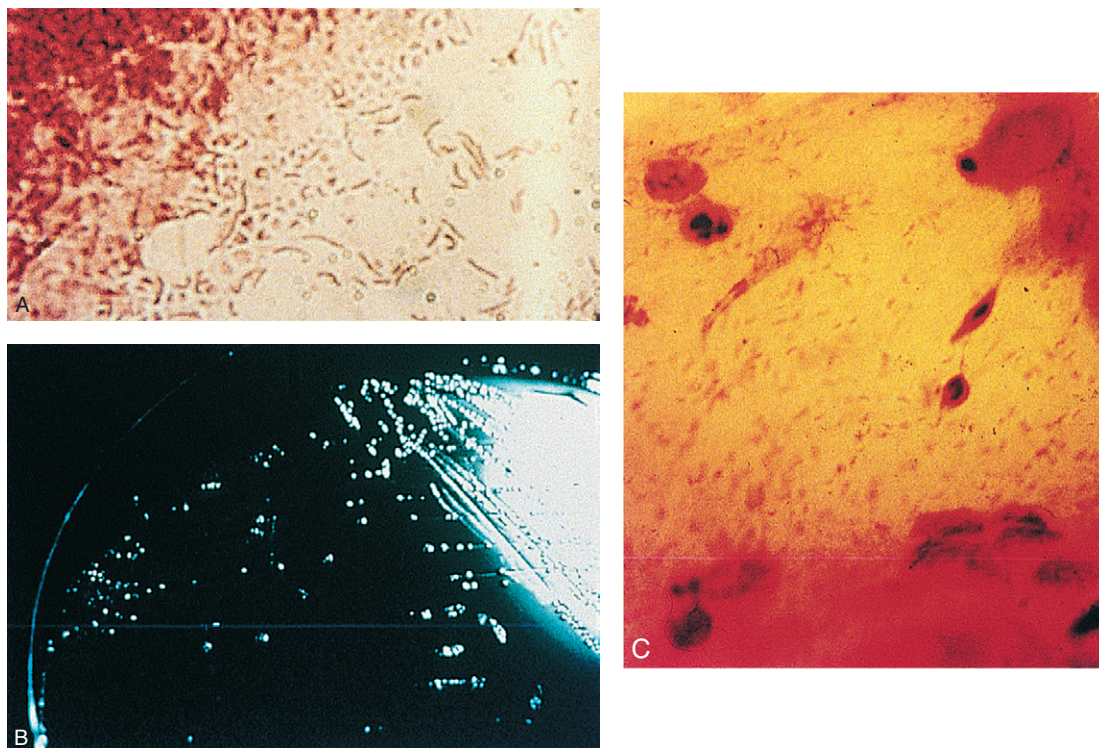


Fig. 34.3 **A**, Microscopic morphology of *Helicobacter pylori* Gram stained from a colony. **B**, Gray translucent *H. pylori* colonies grown on charcoal cefoperazone deoxycholate agar culture medium. **C**, Gram stain on gastric mucus. (Courtesy American College of Gastroenterology and DiaSorin, Stillwater, MN.)

Other bacterial pathogens

Aeromonas spp. and *Plesiomonas shigelloides* have been associated with a watery diarrhea. *Edwardsiella tarda*, associated with fish and shellfish, is a rare cause of gastroenteritis. Organisms responsible for sexually transmitted infections can also cause GI disease. *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Treponema pallidum* subsp. *pallidum*, and herpes simplex virus may all cause a proctitis with loose stools and pain on defecation, usually in patients infected through receptive anal intercourse.

Case check 34.7

Campylos is Greek for "curved," and *bacter* means "rod," thus giving this common curved, gram-negative rod frequently seen on direct Gram-stained smear of stool its name. Other causes of bacterial diarrhea include *Salmonella* spp., *Shigella* spp., *E. coli*, *Vibrio* spp., *Y. enterocolitica*, *C. difficile*, and *L. monocytogenes*.

Parasitic pathogens

Case study

A 21-year-old male patient recently spent 2 weeks backpacking through the Appalachian mountains. One week after his return, he developed cramping abdominal pain, increased flatulence, and diarrhea. Otherwise, he felt well and had no other symptoms. Stool examination did not reveal any RBCs or WBCs, but did show cysts consistent with *G. duodenalis*, and antigen detection confirmed the diagnosis. He was successfully treated with metronidazole.

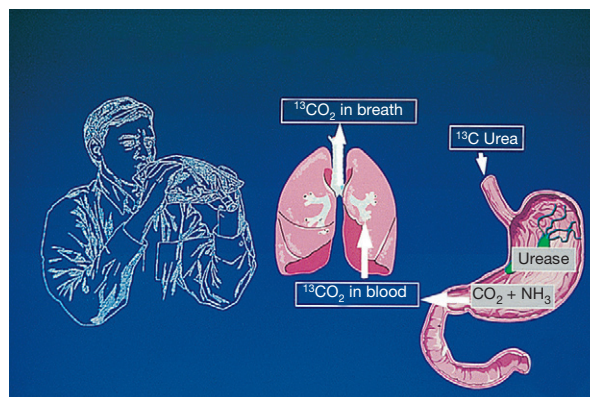


Fig. 34.4 The urea breath test. (Courtesy American College of Gastroenterology and DiaSorin, Stillwater, MN.)

Parasitic infections account for a small percentage of diarrheal illnesses in the United States. They frequently have a longer incubation period than bacterial and viral infections and have a longer clinical course. The diagnosis is based on microscopic examination of the stool or by antigen detection and multiplex nucleic acid methods for some of the parasites.

Giardia duodenalis

Probably the most commonly identified intestinal parasite in the United States, *G. duodenalis*, is usually acquired by ingestion of contaminated water or via person-to-person spread. Because the organism is found in mountain streams, campers and others who engage in outdoor activities are at risk of

infection if they drink contaminated water. After an incubation period of 1 to 2 weeks, patients develop nausea, vomiting, flatulence, cramping, and diarrhea. Because the organism is not invasive, patients typically do not have fever or fecal leukocytes on examination. Stool examination reveals a characteristic trophozoite and/or cyst. The yield of finding the parasite is higher if duodenal aspirates are obtained through endoscopy or the string test (a capsule contains a string that is swallowed and later retrieved by pulling through the oral cavity). Antigen detection and nucleic acid amplification tests are available.

Entamoeba histolytica

Amebiasis from *E. histolytica* (Fig. 34.5) can cause GI and disseminated disease. Because of the invasive nature of *E. histolytica*, the clinical presentation is characterized by fever and bloody diarrhea. However, the range of illness is broad, and infected patients may present with only mild, watery diarrhea, which complicates the diagnosis. Trophozoites and cysts may be identified in the stool. Antigen detection, nucleic acid amplification, and serologic tests for diagnosis are also available. The organism is morphologically similar to the nonpathogenic *Entamoeba dispar*, *Entamoeba moshkovskii*, and *Entamoeba bangladeshi*, also found in the intestinal tract, which has led to much confusion when estimating the true frequency of the disease. The trophozoites of *E. histolytica* can migrate to the liver and cause a liver abscess.

Cryptosporidium parvum and Cryptosporidium hominis

In otherwise healthy individuals, *Cryptosporidium parvum* and *C. hominis* cause an illness characterized by abdominal cramping, watery diarrhea, vomiting, fever, headache, and anorexia. Symptoms generally last about 10 days but may recur after initial abatement. Significant differences exist in clinical manifestations among different *Cryptosporidium* spp. and *C. hominis* subtypes in pediatric cryptosporidiosis.

This organism is resistant to chlorine, so public swimming pools can be the source of an outbreak. In fact, during the summer of 2007, Utah experienced a statewide outbreak of *Cryptosporidium* infection linked to recreational water venues. Approximately 5700 cases were identified. Other sources of exposure include fecally contaminated food or water, international produce, and person-to-person spread. Several

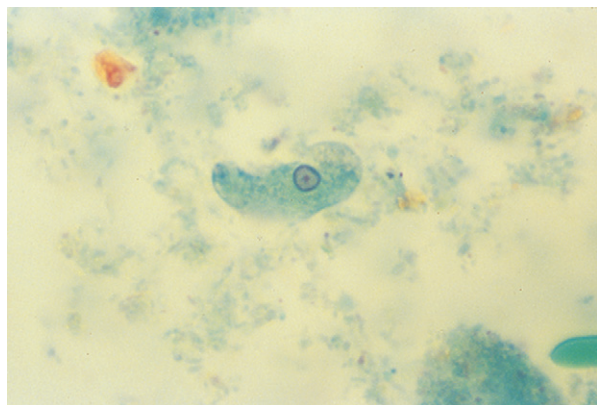


Fig. 34.5 *Entamoeba histolytica* trophozoites (trichrome-stained smear).

outbreaks have been associated with potable water in developing countries. In developing countries, the most common presentation is diarrhea in children. These organisms are acid fast; therefore they can be detected in stool specimens with this staining technique. However, today, they are generally detected by antigen detection or polymerase chain reaction-based assays.

Cyclospora cayetanensis and Cystoisospora belli

Cyclospora cayetanensis has been associated with outbreaks of diarrhea from contaminated water, food, and produce. Illness in the immunocompetent patient generally consists of an acute onset of watery diarrhea, which usually lasts for about 10 days. Patients also may have a prodromal syndrome of myalgias and arthralgias; other symptoms include cramping, fever, profound fatigue, nausea, and vomiting. In healthy individuals, diarrheal disease from *Cystoisospora belli* (formerly *Isospora belli*) is similar to other parasitic infections. Most cases are self-limiting and consist of watery diarrhea and abdominal cramps. In the United States, most cases are seen in travelers returning from endemic areas. Antigen detection assays are not commercially available and serologic tests have minimal value. Some in-house nucleic acid amplification tests have been described. Diagnosis relies on detection of oocysts in unstained or modified acid fast–stained stool samples.

Microsporidia

The fungi *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis* are members of the order Microsporidia. These pathogens can cause illness similar to that caused by *C. parvum* and *C. cayetanensis*. Disease ranges from an asymptomatic to self-limiting diarrhea in healthy individuals. This organism is an important cause of diarrhea in immunocompromised individuals, particularly in patients with AIDS. Special staining of stool specimens is required to observe these organisms under light microscopy; their small size, 1.5 to 4.0 μm , makes detection difficult. Occasionally, they will stain acid fast.

Case check 34.8

Suspect a parasitic cause of infection if the patient has an epidemiologic history of foreign travel or activities such as hiking and a clinical picture consistent with a mild, chronic diarrhea. Parasites can cause severe disease in immunocompromised patients. Diagnosis is often made by direct examination of the stool with special stains, such as acid-fast stains, and antigen testing.

Other parasitic infections

In addition to the protozoal parasitic infections mentioned earlier, there are many other organisms that can cause human disease, most of which are rare in the United States. Some of these organisms include *Ascaris lumbricoides* (common roundworm), *Strongyloides stercoralis*, *Ancylostoma* and *Necator* spp. (hookworms), *Trichuris trichiura* (whipworm), and *Enterobius vermicularis* (pinworm). Others include *Capillaria philippinensis*, which is usually found in Thailand, Iran, Egypt, and the Philippines, and *Trichinella spiralis*, which is acquired by eating poorly cooked pork. *Anisakis* infection is associated with

eating contaminated sushi or sashimi. The parasitic organisms listed here are a sample of the human parasites. Refer to [Chapter 28](#) for a more detailed discussion.

Diarrhea in special circumstances

Toxic agents of food poisoning

Case study

A 32-year-old female patient ate a fish dinner at a restaurant. Before she returned home from the restaurant, she began to feel warm. She developed headache and abdominal cramping with some diarrhea and was generally flushed. Symptoms continued for a few hours and then abated.

A foodborne outbreak is defined as the occurrence of GI or neurologic symptoms within 72 hours of ingesting a contaminated meal. The most commonly identified bacterial agents involved in foodborne outbreaks are *Salmonella* spp., *C. jejuni*, *S. aureus*, *Clostridium botulinum*, and *C. perfringens*. *G. duodenalis*, *Cryptosporidium*, and *Cyclospora* have also been associated with outbreaks, usually linked to contaminated fruits and vegetables.

Chemical intoxications are often associated with fish consumption. The case above is the typical presentation of **scombroid**. This syndrome, which consists of flushing, headache, and crampy abdominal pain with diarrhea, is caused by ingesting contaminated fish (often tuna, mackerel, or yellow jack). The tissues of the fish contain histamine and enzyme inhibitors, which are responsible for the symptoms. The symptoms usually start within 1 hour of ingestion and usually last only several hours.

Ciguatera is caused by a toxin, ciguatoxin, produced by dinoflagellates. This toxin accumulates in fish as it passes up the food chain. Most cases are associated with eating snapper, sea bass, grouper, or barracuda. The symptoms include diarrhea, abdominal pain, weakness, paresthesias, and headache. Symptoms often begin within 1 to 2 hours of eating the contaminated fish and may progress to respiratory failure and hypotension. Because an antitoxin is not available, patients are treated supportively.

Paralytic shellfish poisoning is associated with eating contaminated clams, mussels, and scallops. Usually occurring during the summer months, paralytic shellfish poisoning produces a syndrome similar to ciguatera. One of the deadliest toxins, tetrodotoxin, which can be found in the puffer fish, causes death in more than 50% of patients exposed. The contaminated food appears normal in each of these cases of toxin-mediated food poisoning. Prevention is aimed at locating and removing sources of contaminated fish. [Table 34.5](#) outlines several of the most common foodborne pathogens.

Returning travelers

Traveler's diarrhea is a common problem in patients visiting developing countries. Although most cases are often mild and self-limiting, it can lead to significant morbidity and disrupt the traveler's itinerary. Traveler's diarrhea usually

occurs within the first 2 weeks of travel. Other symptoms include malaise, abdominal pain, fever, nausea and vomiting, and occasionally blood in the stool. The CDC has classified the risk of developing the disease by geography, as shown in [Fig. 34.6](#). The destinations holding the highest risk for traveler's diarrhea include Asia, Africa (excluding South Africa), Central America, and Mexico.

The infectious causes of traveler's diarrhea are multiple, including many of the bacterial, viral, and parasitic organisms already described. The most common cause of traveler's diarrhea is ETEC, which causes approximately 50% of cases. Other causes include EAEC, *Salmonella*, *C. jejuni*, and *Shigella* spp. Rotaviruses are the most common viral GI pathogen. Parasitic infections are less commonly associated with typical traveler's diarrhea. However, travelers to certain areas are at a higher risk for such infections; for example, travelers to mountainous regions, especially if they are camping and drinking spring water, are at increased risk for *G. duodenalis* infection. The two geographic areas with the highest rates of *Giardia* infection are Nepal and St. Petersburg in Russia. For up-to-date information regarding management of traveler's diarrhea, refer to the CDC website. Currently, prophylaxis is not recommended universally; however, for individuals in whom diarrhea may have severe consequences, bismuth subsalicylate (Pepto-Bismol), two tablets twice daily, has been proven to decrease the frequency of traveler's diarrhea. Travelers are often provided with a 5-day course of azithromycin for self-treatment. Fluoroquinolones are no longer recommended empirically because of growing resistance among bacterial GI pathogens.

Immunocompromised hosts

Modern medicine has increased the number of people who are immunocompromised because better treatments for chronic diseases are available. Failing organs can now be replaced, and those who have congenital immunodeficiency are living longer. Immunocompromised hosts include individuals with AIDS; cancer patients, especially while receiving chemotherapy; those who have received a solid organ or bone marrow transplant; and those with chronic diseases such as rheumatoid arthritis that require continuous immunosuppression.

All of the pathogens described earlier can cause diarrhea in the immunocompromised host. Usually, the clinical course is more severe and of longer duration. These patients are also at risk for developing infections with opportunistic organisms. **Opportunistic pathogens** generally do not cause disease in healthy individuals, but they cause significant disease in susceptible hosts. In addition to infectious diarrhea, immunocompromised patients may also develop diarrhea caused by drugs, graft-versus-host disease, or the disease process itself, such as in HIV enteropathy.

Mycobacterial disease, which typically causes pulmonary disease, can spread and cause infection of the GI tract in immunocompromised individuals. The two most common mycobacteria causing disease in these hosts are *Mycobacterium tuberculosis* and *Mycobacterium avium* complex. In HIV-infected patients, *Mycobacterium avium* complex is rarely seen unless the CD4 cell count drops below 50/ μ L. Patients may present with abdominal pain and diarrhea, which sometimes turns bloody. Other symptoms such as weight loss, fevers, and night sweats are often present.

Table 34.5 Compendium of common foodborne diseases

Average incubation period	Organism	Average duration	Implicated foods	Typical symptoms	Comments
2–16 hours	<i>Bacillus cereus</i>	1 day	Boiled and fried rice, meats, vegetables	Nausea, vomiting, (emetic) abdominal cramping, watery diarrhea	Produces two toxins: one emetic form that causes nausea and vomiting within hours, and one diarrheic form; common year-round; isolation of large numbers of bacteria from implicated foods and patient stool
6–72 hours	<i>Vibrio parahaemolyticus</i>	3 days	Shellfish	Abdominal pain, vomiting, fever, watery diarrhea	Blood sometimes in stool; common in spring, summer, and fall in coastal states; stool culture using TCBS medium recommended
6–72 hours	<i>Vibrio cholerae</i>	3–7 days	Seafood, water	Rice water stools, severe diarrhea, no fever	No blood or mucus in stool; mechanism of action, in vivo enterotoxin production; no tissue invasion; stool culture using TCBS medium recommended
<8 hours	<i>Staphylococcus aureus</i>	<1 day	Egg salad, meat, poultry, pastries	Abrupt onset of nausea, abdominal pain and projectile vomiting, infrequent diarrhea, no fever	Mode of infection is ingesting preformed enterotoxin in foods; common in summer; ELISA or reverse passive latex agglutination enterotoxin test
8–22 hours	<i>Clostridium perfringens</i>	1 day	Beef, poultry, gravy, fish	Abdominal cramping, watery diarrhea; vomiting and fever uncommon	In vivo enterotoxin production; unlike <i>Staphylococcus aureus</i> , viable organisms must be ingested for disease to occur; common in fall, winter, and spring
12–48 hours	<i>Salmonella</i> sp.	3 days	Eggs, dairy products, fowl, beef	Fever, abdominal cramping, diarrhea, mild vomiting	WBCs in stool; common in summer; culture and serologic identification
16–48 hours	<i>Yersinia enterocolitica</i>	1 day to 4 weeks	Milk, pork	Fever, severe abdominal pain, diarrhea	WBCs and RBCs in stool; common in winter
18–36 hours	<i>Clostridium botulinum</i>	Weeks to months	Vegetables, fruits (canned foods), fish, honey (infants)	Nausea, vomiting, diarrhea, paralysis	Mode of infection is ingesting preformed neurotoxin; common in summer and fall
24–72 hours	<i>Shigella</i> spp.	3 days	Egg and tuna salads, lettuce, milk	Fever, abdominal cramping, diarrhea, occasional vomiting	WBCs, RBCs, and mucus in stool; tissue invasion common mechanism of action; common in summer; culture and serologic identification
24–72 hours	Enterotoxigenic <i>Escherichia coli</i>	3 days	Fruits, meats, pastries, salads	Abdominal cramping, watery diarrhea, no vomiting or fever	In vivo enterotoxin; major cause of traveler's diarrhea; year-round distribution; patient history includes travel to Mexico and other developing countries
24–72 hours	Enterohemorrhagic <i>E. coli</i>	3 days	Undercooked ground beef, apple cider	Watery diarrhea progressing to bloody diarrhea, abdominal cramping, no fever or vomiting	Implicated Shiga-toxin producing <i>E. coli</i> ; organisms disappear rapidly from stool; culture of sorbitol-negative <i>E. coli</i> from stool using SMAC plate recommended

ELISA, Enzyme-linked immunosorbent assay; RBCs, red blood cells; SMAC, sorbitol MacConkey; TCBS, thiosulfate–citrate–bile salts–sucrose; WBCs, white blood cells.

Diagnosis is often made based on positive blood cultures in disseminated disease.

Cytomegalovirus (CMV) is an opportunistic virus that can lead to diarrhea. CMV can infect many organs, including the retina, lungs, and GI tract. It can cause esophageal ulcers, leading to painful swallowing, and colitis. Typical

symptoms consist of watery diarrhea, abdominal pain, and blood in the stool. Fever is often present. The diagnosis is established with colonoscopy; findings include patchy erythema, edema, erosions, and ulcerations. Intracytoplasmic inclusion bodies are seen on microscopic evaluation of tissue biopsy samples.

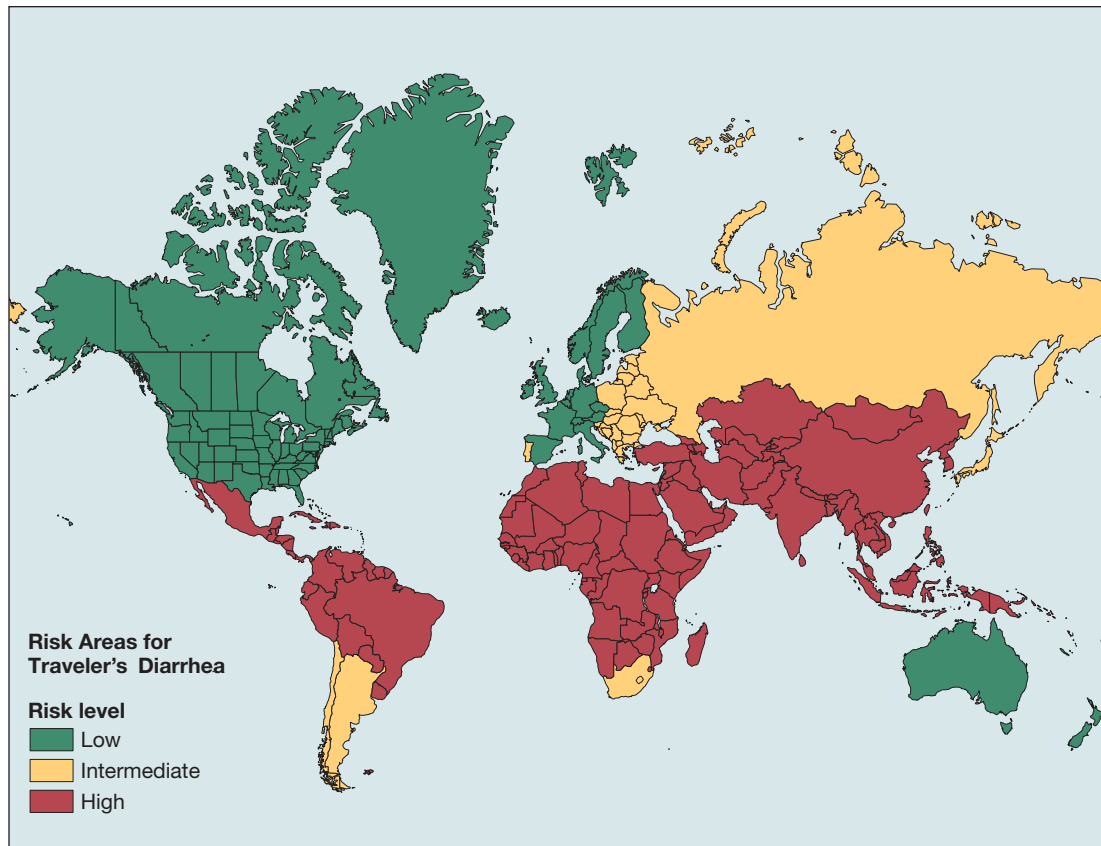


Fig. 34.6 Geographic distribution of risk for traveler's diarrhea.

GI histoplasmosis should be considered in HIV-infected patients living in endemic regions who present with fever, abdominal pain, weight loss, diarrhea, GI obstruction, abdominal mass, hepatosplenomegaly, or intestinal perforation. Diagnosis is made by stool culture for fungi, urine *Histoplasma* antigen testing, or pathologic examination of GI tissue specimens.

Before the introduction of highly active antiretroviral therapy (HAART), *Cryptosporidium* spp. caused diarrhea as the presenting illness in up to 50% of HIV-infected patients. Morbidity is now drastically decreased in the era of HAART, but *Cryptosporidium* spp. are still a problem in countries with limited access to HAART. Diagnosis is made by detecting oocytes with modified acid-fast stain of the stool or a direct fluorescent antibody testing. Modified acid-fast stains also detect *Cyclospora*, *Cystoisospora*, and microsporidia.

Strongyloidiasis is a parasitic infection caused by the intestinal nematode *Strongyloides stercoralis* and is endemic in tropical and subtropical regions of the world. Most patients are asymptomatic, but immunocompromised patients may develop hyperinfection syndrome caused by an increased number of larvae. Hyperinfection syndrome may present with dyspnea, pleuritic pain, and hemoptysis as the larvae migrate to the lungs, penetrate the alveoli, ascend the tracheo-bronchial tree, and are then swallowed. Diagnosis is made by stool testing for ova and parasites, complete blood count to check for eosinophilia, and serologic testing.

The pathogens discussed are only a few of the many organisms that cause disease in immunocompromised hosts. Thus the diagnostic workup in these patients is often more extensive. Guided by the history and epidemiologic clues, such as recent travel, diagnostic testing usually includes stool cultures for

bacterial and fungal organisms, ova and parasite stool examinations, antigen detection or nucleic acid amplification for certain parasites and viruses, special staining, and serologic testing for particular organisms. Endoscopy is often done in severe or recurrent disease for a definitive diagnosis. If infectious causes are ruled out, noninfectious causes should be investigated.

Laboratory diagnosis of gastrointestinal pathogens

Specimen collection and handling

Stool specimens should be transported to the laboratory shortly after collection, avoiding refrigeration if possible. Preservatives must be avoided if bacterial cultures are ordered. However, if the stool is to be examined for ova and parasites, it should be transported in media containing preservatives such as polyvinyl alcohol or formalin. If rectal swabs for bacterial culture are to be processed, Cary-Blair or a similar transport medium should be used. Due to high sensitivity and specificity and rapid turnaround time, multiplex polymerase chain reaction has replaced routine culture in many large microbiology laboratories.

Direct microscopic examination

Microscopic examination of the stool may reveal WBCs in cases of inflammatory diarrhea (e.g. *Salmonella*, *Shigella*, *Yersinia*, *Campylobacter*, EIEC, and various *Vibrio* spp.).

RBCs may be present because of intestinal wall bleeding. The bacteria may be visible on direct microscopic examination of the stool. The presence of gram-negative, curved rods with a “seagull wing” appearance (Fig. 34.7) suggests *Campylobacter* or *Vibrio* infection. If wet mount or hanging drop preparations are performed in a patient with *C. jejuni* infection, characteristic darting motility may be seen. *E. histolytica* may also be seen on direct examination in patients with bloody diarrhea.

Culture

Selective and differential culture media are commonly used to attempt to identify bacterial pathogens in stool. Selective media contain antimicrobials or chemicals that limit the growth of normal bacterial biota and enhance the growth of pathogenic bacteria. The differential aspect of the medium often allows differentiation of bacterial species based on colony morphology; the differences in colony appearance are usually a result of different biochemical characteristics of the organisms. Table 34.6 lists some of the selective media used to isolate GI pathogens.

Campylobacter jejuni

C. jejuni grows best at 42°C in an atmosphere of reduced oxygen concentration (5% to 10%), which makes them microaerophilic organisms. Laboratories may have special gas supplies and culture chambers to provide an optimal atmosphere for culturing this organism. *C. jejuni* colonies form a characteristic morphology described as “running” or “wet looking” on growth media. Microscopic examination of the organisms shows characteristic curved, gram-negative rods (see Fig. 34.7). This appearance helps differentiate *C. jejuni* from *Pseudomonas aeruginosa*, which can grow under similar environmental conditions.

Salmonella

In patients with typhoid fever, blood cultures are most likely to be positive in the first week of infection, whereas stool cultures are more likely to be positive in the third and fourth weeks of infection. In patients with nontyphoid *Salmonella* gastroenteritis, the organisms may be recovered from stool. Routine microbiologic media such as sheep blood agar (SBA)

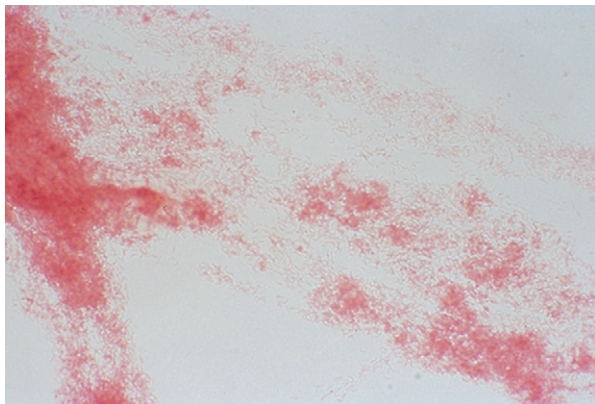


Fig. 34.7 Gram stain of *Campylobacter* colony showing the typical microscopic morphology described as seagull wings.

and MacConkey (MAC) agar can be used. Highly selective media such as Hektoen enteric (HE) agar (Fig. 34.8) and xylose-lysine-deoxycholate (XLD) agar may also be used.

Shigella species

These bacteria are fragile and do not survive well outside the human host for long periods. Culture recovery is enhanced by rapid transport of the specimen to the microbiology laboratory and prompt processing of the specimen. Media that can successfully recover *Salmonella* spp. are also useful for the recovery of *Shigella* spp. (Fig. 34.9).

Escherichia coli

E. coli is a normal inhabitant of the human GI tract. Pathogenic *E. coli* have the same appearance as commensal *E. coli* in culture media. A few diagnostic tests help differentiate pathogenic from nonpathogenic *E. coli*. For example, many EHEC will not ferment sorbitol. These bacteria can be differentiated from sorbitol-fermenting *E. coli* with the use of a differential medium such as sorbitol MAC agar (Fig. 34.10). EIEC has morphology and biochemical reactions similar to those of *Shigella* spp.

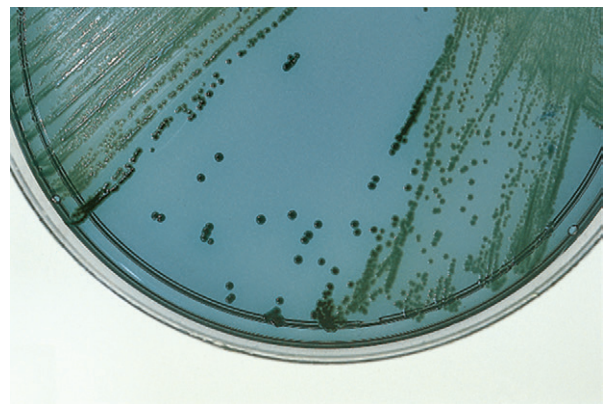


Fig. 34.8 *Salmonella* colonies growing on Hektoen enteric agar showing black centers resulting from the production of hydrogen sulfide.

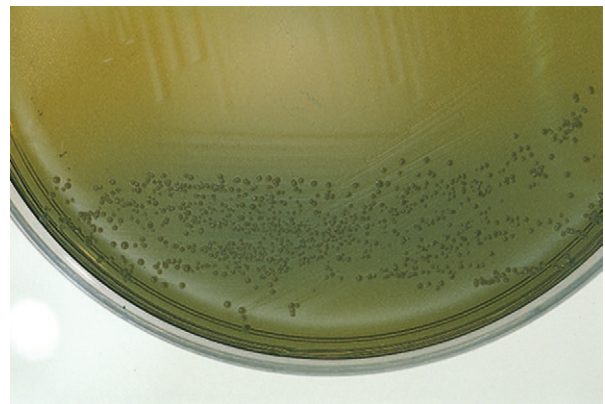


Fig. 34.9 *Shigella* colonies growing on Hektoen enteric agar showing clear green colonies.

Table 34.6 Selective media commonly used to recover diarrheal agents

Culture medium	Purpose	Characteristic morphology	
		Pathogens	Colon biota
MacConkey (MAC) agar	To recover Enterobacterales and other nonfastidious, gram-negative bacilli; inhibits gram-positive organisms and some fastidious gram-negative bacilli	<i>Salmonella</i> , <i>Shigella</i> spp. (with few exceptions); organisms appear clear and colorless	Lactose fermenters, such as <i>Escherichia coli</i> , <i>Klebsiella</i> spp., <i>Enterobacter</i> spp., and certain <i>Citrobacter</i> spp., appear dark pink to red. Delayed or slow lactose fermenters, such as <i>Citrobacter</i> spp., <i>Serratia</i> spp., and <i>Hafnia</i> spp., appear colorless in 24 hours and slightly pink after 24–48 hours; non-lactose fermenters, such as <i>Proteus</i> spp., <i>Providencia</i> spp., and <i>Morganella</i> spp., appear clear and colorless.
Hektoen enteric (HE) agar	Highly selective medium to recover primarily <i>Salmonella</i> and <i>Shigella</i> spp.; inhibits common colon biota; contains indicators to detect hydrogen sulfide (H ₂ S) production	<i>Salmonella</i> appears green to blue-green with black centers because of H ₂ S production; <i>Shigella</i> spp. appear green without black centers because they do not produce H ₂ S	Lactose fermenters, such as <i>E. coli</i> , are slightly inhibited and appear orange to salmon pink; <i>Proteus</i> spp. are slightly inhibited; small, clear colonies with black centers may appear.
Xylose-lysine-deoxycholate (XLD) agar	Differential and selective medium to isolate <i>Salmonella</i> and <i>Shigella</i> spp. from stool; inhibits most colon biota and most gram-positive bacteria; certain <i>Shigella</i> spp. (<i>S. dysenteriae</i> and <i>S. flexneri</i>) may be slightly inhibited	<i>Salmonella</i> appear red with black centers because of production of H ₂ S; <i>Salmonella</i> does not ferment lactose or sucrose but does ferment xylose, which is essential in decarboxylating lysine to cause the acid pH (yellow from sucrose fermentation) to revert to an alkaline pH (red from lysine decarboxylation) <i>Shigella</i> spp. do not ferment any of these carbohydrates and appear red or clear	Enterobacterales that may not be completely inhibited, such as <i>Proteus vulgaris</i> , appear yellow (from sucrose) with black centers; <i>Citrobacter freundii</i> , which produces H ₂ S, appears yellow with black centers because of inability to decarboxylate lysine; other intestinal biota that may grow will ferment one or all of the carbohydrates in this medium, resulting in yellow colonies.
<i>Campylobacter</i> blood agar (CAMPY-BA)	Enrichment-selective medium primarily to isolate and cultivate <i>Campylobacter</i> spp. from stool	<i>Campylobacter jejuni</i> appears pinkish gray, moist, and runny when incubated at 42° C	
Cefsulodin-irgasan-novobiocin (CIN) agar	Selective medium primarily to isolate and recover <i>Yersinia enterocolitica</i> and <i>Aeromonas</i> spp.; <i>Plesiomonas shigelloides</i> may also be recovered; inhibits most gram-positive cocci, except for enterococci, and most gram-negative bacilli, particularly the Enterobacterales	<i>Yersinia enterocolitica</i> produces colonies that look like bull's eyes; center is red and periphery appears colorless; <i>Aeromonas</i> species also ferment mannitol present in the medium, like <i>Yersinia</i> ; <i>P. shigelloides</i> does not	Except for <i>Pseudomonas aeruginosa</i> , <i>Citrobacter</i> , and <i>Serratia</i> , most colon biota are inhibited.
Thiosulfate–citrate–bile salts–sucrose (TCBS) agar	Highly selective medium to recover <i>Vibrio</i> spp., including <i>Vibrio cholerae</i> , from stool and food; inhibits most colon biota because of the high pH (preferred by vibrios) and high bile salts content; <i>Aeromonas</i> spp. may be recovered from this medium	TCBS contains sucrose, so sucrose-fermenting <i>Vibrio</i> spp. such as <i>V. cholerae</i> and <i>V. alginolyticus</i> produce yellow colonies; nonsucrose fermenters, such as <i>V. parahaemolyticus</i> and <i>V. vulnificus</i> , produce blue-green colonies	Inhibitory to most colon biota, except for occasional <i>Pseudomonas</i> isolates, which may also appear blue-green.
Cycloserine-cefoxitin-fructose agar (CCFA), anaerobic incubation required	Selective medium to isolate primarily <i>Clostridioides difficile</i> from stool of patients suspected of having antibiotic-associated diarrhea or pseudomembranous colitis; inhibits most colon biota, both gram-positive and gram-negative bacteria	<i>C. difficile</i> appears yellow because of fructose fermentation	Colon biota are inhibited.
Sorbitol MacConkey (SMAC) agar	Differential medium to detect sorbitol-negative <i>Escherichia coli</i> ; contains sorbitol instead of lactose	<i>E. coli</i> O157:H7 appears colorless; does not ferment sorbitol	Most appear pink.

Yersinia species

Yersinia spp. grow well at cooler temperatures (25°C). The use of certain selective media, such as cefsulodin-irgasan-novobiocin (CIN) agar, allows for rapid isolation of these species. *Y. enterocolitica* forms colonies with a deep red center and transparent periphery. Recovery of the organism is increased by placing fecal samples in isotonic saline and keeping them at 4°C before inoculation onto the selective medium.

Vibrio species

Vibrio spp. require highly selective media for recovery. If these pathogens are suspected, the initial stool specimen should be transported to the laboratory in Cary-Blair medium or a similar transport medium. The sample can then be inoculated on thiosulfate–citrate–bile salts–sucrose (TCBS) agar for maximal yield. This medium not only inhibits the normal colonic biota but also differentiates sucrose-fermenting from non-sucrose-fermenting *Vibrio* spp. (Fig. 34.11). The salt requirement for growth may also help differentiate *V. cholerae*

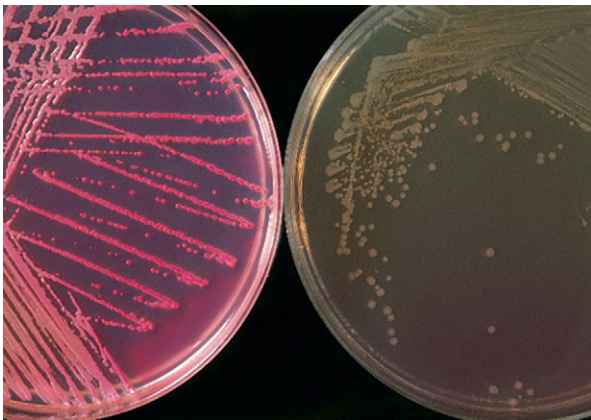


Fig. 34.10 Left, *Escherichia coli* O157:H7 growing on MacConkey agar. Right, *E. coli* O157:H7 on sorbitol MacConkey agar. *E. coli* O157:H7 does not ferment sorbitol, whereas most other *E. coli* serotypes do ferment sorbitol.

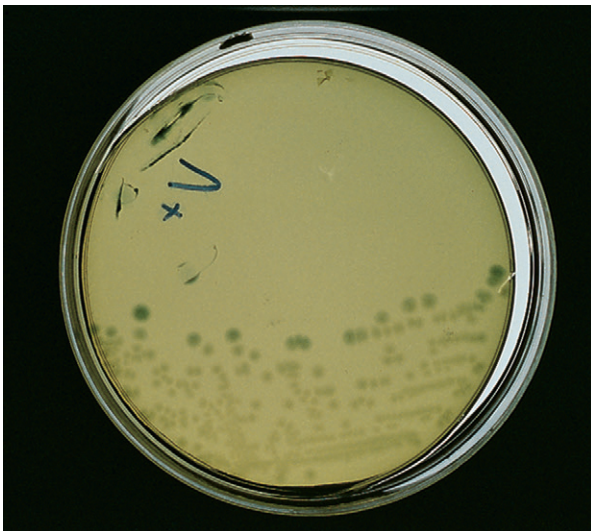


Fig. 34.11 *Vibrio vulnificus* growing on thiosulfate–citrate–bile salts–sucrose agar. *Vibrio vulnificus* is a nonsucrose-fermenting vibrio.

from more halophilic *Vibrio* spp. In certain areas, especially in regions where cholera is endemic, antisera are available to identify outbreak strains of *V. cholerae*.

Clostridioides difficile

C. difficile produces yellow, ground-glass colonies on cycloserine-cefoxitin-fructose agar. Culture is infrequently used to diagnose this pathogen because patients may be colonized with non-toxin-producing strains. Instead, stool specimens are often examined for the presence of the *C. difficile* toxins.

Treatment and prevention of diarrhea

The most important factor to consider when treating a patient with acute diarrhea is his or her hydration status. Volume depletion and electrolyte derangements are major causes of morbidity and mortality from diarrheal illnesses. Because patients lose electrolytes in diarrheal stools, rehydration cannot occur with water alone. The best hydration solution contains both glucose and sodium. Although most patients can be treated effectively with oral rehydration, some require IV infusion of an isotonic solution (e.g., 0.9% saline, 5% dextrose in 0.9% saline, Ringer's lactate).

Antimicrobial therapy is ineffective against the viral causes of gastroenteritis. Patients with viral gastroenteritis should be given supportive care, with adequate hydration. Measures should be taken to prevent the spread of the pathogen to others. In severe cases, antimicrobial agents may be used against some bacterial pathogens, such as *Salmonella*. However, in several cases, this may be associated with a longer period of shedding of the organism in the stool, placing other people at risk of contracting the disease. Treating infections caused by EHEC with antimicrobial agents may increase the chance of developing HUS.

Antimicrobial therapy can be effective in shortening the duration of illness associated with some of the intestinal parasites. Antidiarrheal medications such as diphenoxylate with atropine (Lomotil) or loperamide can be considered for patients with severe symptoms. However, antimotility agents are not recommended for invasive diarrhea. Studies show conflicting data, but these agents may increase the severity of invasive disease, likely because of an increased contact time of the pathogen with the intestinal wall. Antimotility agents are generally used for the treatment of enterotoxin-mediated diarrhea, viral gastroenteritis, or traveler's diarrhea. Bismuth subsalicylate (Pepto-Bismol) can be effective in shortening the course of traveler's diarrhea, especially when caused by ETEC. People traveling to areas with a high prevalence of GI pathogens should carry with them a short course of a macrolide such as azithromycin for self-treatment of traveler's diarrhea and antimotility agents. Although this may be effective in reducing the duration of illness, most cases of traveler's diarrhea are self-limiting, even without therapy.

Travelers to high-risk areas should be advised to drink only bottled beverages and avoid consuming ice and undercooked food. Examples of high-risk foods include salads, fruits, dips, and raw or partially cooked fish or shellfish. Foodborne illnesses are avoided by thoroughly cooking all meats and poultry and by thoroughly washing all fruits and vegetables with potable water. Ideally, fruits and vegetables should be

peeled before use. To prevent secondary infections, patients with acute gastroenteritis should practice careful handwashing and avoid preparing food for others. The CDC maintains a website with the most recent recommendations (<https://wwwnc.cdc.gov/travel/page/yellowbook-home-2020>).

Two pathogens have been effectively targeted by a vaccine to help decrease the burden of disease: rotavirus and *Salmonella* Typhi. Two vaccines are approved for use in the United States. The CDC recommends that travelers to a country where typhoid is common should consider being vaccinated. The vaccine's efficacy in preventing diarrheal illness ranges from 50% to 80%. However, immunity produced by the vaccine wanes after a few years.

POINTS TO REMEMBER

- Factors that predispose patients to diarrheal illnesses include lack of clean drinking water, travel to endemic countries, history of GI disease, immunocompromised state, and medication intake.
- Travel history and a detailed dietary history provide significant information when determining the cause of diarrheal disease. Certain foods such as meat, shellfish, and poultry serve as vehicles of foodborne disease.
- Foodborne diseases are transmitted and acquired by ingestion of contaminated food and beverages.
- Clinical presentations can be characterized as enterotoxin-mediated diarrhea, invasion of bowel mucosa, or invasion with lymphatic or metastatic spread of infection.
- Food poisoning may be caused by chemical intoxications from fish or shellfish consumption, as well as bacterial toxins.
- Appropriate laboratory diagnosis may include the use of direct microscopy and selective culture media to recover and identify the suspected causative agent.

LEARNING ASSESSMENT QUESTIONS

1. What are the major host defense mechanisms located in the stomach? The small bowel? The colon?
2. What are the key elements to obtain in the history when interviewing a patient with diarrheal illness?
3. What initial laboratory findings aid in the diagnosis of acute diarrhea?
4. Which viruses, usually not associated with diarrheal illness in immunocompetent patients, may result in diarrhea in patients with acquired immunodeficiency syndrome (AIDS)?
5. How is the workup of diarrhea in an immunocompromised patient different from that in a healthy host?
6. Which parasite may cause a bloody diarrhea and disseminated infection sometimes resulting in liver abscesses?
7. What are some toxin-mediated forms of illness associated with fish and shellfish?
8. What are some life-threatening complications associated with bacterial diarrheal infections?
9. Which diarrheal infection is associated with prior antimicrobial use?
10. Which serotypes of *Vibrio cholerae* are associated with epidemic diarrhea?
11. How can travelers avoid diarrhea?
12. Which virus causes an acute self-limiting diarrheal illness that is highly contagious through contaminated food, water, fomites, or person-to-person contact and is associated with vomiting and low-grade fever?
 - a. Astroviruses
 - b. Rotaviruses
 - c. Norovirus
 - d. Enteric arenaviruses
13. A male patient and his family drove to the Florida coast and ate an oyster dinner. A couple of days later, the patient arrives at the emergency department with a fever, bullous skin lesions, and diarrhea. He is extremely ill and appears septic. The microbiologist notices curved, gram-negative rods on the stool Gram-stained smear. What is the most probable organism causing this condition?
 - a. *Yersinia enterocolitica*
 - b. *Vibrio vulnificus*
 - c. *Campylobacter jejuni*
 - d. *Vibrio cholerae*
14. Which diarrheal pathogen can produce a disease that can lead to hemolytic-uremic syndrome?
 - a. Enterohemorrhagic *Escherichia coli* (EHEC)
 - b. Enteroinvasive *E. coli* (EIEC)
 - c. Enteropathogenic *E. coli* (EPEC)
 - d. Enterotoxigenic *E. coli* (ETEC)
15. Although uncommon, which of the following intestinal parasite may cause metastatic disease and produce liver abscesses?
 - a. *Giardia lamblia*
 - b. *Entamoeba histolytica*
 - c. *Microsporidia*
 - d. *Cryptosporidium*
16. A patient who complains of sharp back pain is suspected to have cholecystitis. The patient is taken to surgery, and the infected gallbladder is removed. Exudate from the gallbladder is sent to the laboratory for culture. Which of the following organisms would you try to recover in an infected gallbladder?
 - a. *Escherichia coli* O157:H7
 - b. *Yersinia enterocolitica*
 - c. *Shigella* spp.
 - d. *Salmonella* spp.
17. A 15-year-old female patient is admitted to the hospital because of lower quadrant pain and a fever of 102° F. The patient's white blood cell count is 12,000/mm³ with 75% neutrophils and 6% bands. The clinical diagnosis is presumed acute appendicitis. At surgery, the appendix is grossly normal, but enlarged mesenteric lymph nodes are noted. One of these is excised and sent to the laboratory for culture. Which organism would most likely produce this clinical picture?
 - a. *Vibrio parahaemolyticus*
 - b. *Yersinia enterocolitica*
 - c. *Shigella* spp.
 - d. *Salmonella* spp.
18. A 30-year-old male patient served barbecued chicken for dinner with friends during a Sunday football game. On Tuesday following the event, the patient and a few of his guests develop fever and diarrhea. Microscopic stool

studies show red blood cells, leukocytes, and a very distinct microscopic morphology of the etiologic agent that can be described as “seagull wings.” After several days, the patient and his guests recovered without complications. Based on the preliminary findings, which bacterial agent should you suspect?

- a. *Campylobacter jejuni*
- b. *Yersinia enterocolitica*
- c. *Shigella* spp.
- d. *Salmonella* spp.

19. A 2-year-old female patient who attends a daycare center is admitted to the hospital with a 3-day history of fever, abdominal pain and cramping, watery diarrhea, and vomiting. The stool shows gross blood and pus. Stool culture grows many lactose-negative colonies on MacConkey agar after 24 hours of incubation. Which of the following organisms is the most probable identification of the isolate?

- a. *Providencia stuartii*
- b. *Yersinia enterocolitica*
- c. *Shigella* sp.
- d. *Salmonella* sp.

20. A patient with a history of alcoholism is seen in the emergency department with symptoms of septicemia and bullous skin lesions. He reported ingestion of raw oysters 24 hours earlier. Which of the following organisms should you suspect as the causative agent?

- a. *Shigella sonnei*
- b. *Yersinia enterocolitica*
- c. *Vibrio vulnificus*
- d. *Campylobacter jejuni*

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Infections of the central nervous system

Sumathi Nambiar and Kalavati Suvama^a

CHAPTER OUTLINE

General concepts related to infections of the central nervous system, 890

- Anatomic organization, 890
- Cerebrospinal fluid characteristics, 891
- Host-pathogen relationships, 891

Central nervous system infections, 891

- Meningitis, 891
- Meningoencephalitis and encephalitis, 898

Laboratory diagnosis, 899

- Cerebrospinal fluid transport and analysis, 899
- Culture, 900
- Molecular diagnostics, 901

Bibliography, 903

OBJECTIVES

After reading and studying this chapter, you should be able to:

1. Explain how cerebrospinal fluid (CSF) is produced and distributed in the central nervous system (CNS).
2. Describe the characteristics of normal CSF.
3. Describe the collection, transportation, and processing of CSF samples.
4. For each pathogen associated with meningitis, correlate one host-related risk factor and one virulence-related risk factor.
5. Identify the common bacterial, fungal, and parasitic pathogens associated with brain abscesses.
6. Correlate fungi that typically cause meningitis.
7. Compare the virulence and host factors of two fungi that typically cause intracerebral lesions.
8. Associate two viruses associated with meningitis, encephalitis, and paralysis.
9. Correlate cerebrospinal fluid laboratory results (i.e., glucose, protein, cell counts) with likely agents causing meningitis.

^aThis chapter was prepared by the author in her private capacity. No official support or endorsement by the U.S. Food and Drug Administration is intended or implied.

10. Justify rapid processing of CSF and other specimens from the CNS for the evaluation of infectious agents.

KEY TERMS

Aseptic meningitis

Brain abscess

Central nervous system (CNS)

Cerebrospinal fluid (CSF)

Encephalitis

Meningismus

Meningitis

Meningoencephalitis

Pleocytosis

Primary amebic meningoencephalitis

Tuberculous meningitis

Case in point

A 3-year-old male patient with a recent history of acute otitis media was brought to the emergency department. On examination, he was found to be febrile with an oral temperature of 103°F (39.4°C), reference range 95.9°F (35.5°C) to 99.5°F (37.5°C), and lethargic. No rash was present. A complete blood count revealed leukocytosis with a total leukocyte count of 21,000/μL (reference range 5000 to 10,000/μL) with a left shift. Lumbar puncture revealed a cloudy cerebrospinal fluid (CSF) with a leukocyte cell count of 210/μL (reference range ≤5) with 85% neutrophils. The CSF glucose level was decreased (15 mg/dL), and the CSF protein level was increased (450 mg/dL). The patient was given ceftriaxone intravenously, and the CSF sample was sent to the microbiology laboratory for Gram stain and culture. The Gram-stained smear of the cytocentrifuged CSF revealed a moderate number of intracellular, gram-positive cocci in pairs. Culture of the CSF grew a mucoid strain of *Streptococcus pneumoniae*. Susceptibility testing was performed, and the following minimal inhibitory concentrations (MICs) were obtained: penicillin 2.0 μg/mL, ceftriaxone 0.012 μg/mL, and vancomycin 0.25 μg/mL.

Issues to consider

After reading the patient's case history, consider:

- Host-related risk factors in the patient
- Bacterial, fungal, or parasitic agents that may be involved based on the clinical presentation, and possible sources of infection

- Physical, chemical, and cellular features of CSF
- Specimen collection, transport, and processing for maximum recovery of the causative agent
- Rapid diagnostic methods that can facilitate the presumptive diagnosis and initiation of therapy

Infections of the **central nervous system (CNS)** are serious and potentially life-threatening. These infections may be caused by bacteria, viruses, fungi, or parasites. The primary health care provider arrives at a presumptive diagnosis based on the patient's age, presence of risk factors, clinical presentation, physical examination, local epidemiology of CNS infections, **cerebrospinal fluid (CSF)** analysis, and radiologic studies. The specific causative agent is identified through laboratory testing.

The epidemiology of bacterial meningitis has changed over the decades. Since the introduction of vaccination against *Haemophilus influenzae* type b in the 1980s, there has been a dramatic reduction in the incidence of meningitis caused by this organism. In the United States, *Streptococcus pneumoniae* has become the leading cause of bacterial meningitis among children younger than 5 years of age. With the inclusion of pneumococcal vaccination in the routine immunization schedule for children, universal screening of pregnant women for group B *Streptococcus* (GBS), and the availability of newer meningococcal vaccines, there has been a further reduction in the incidence of bacterial meningitis.

Pneumococci cause over 50% of all cases of bacterial meningitis in the United States. An estimated 3000 to 6000 cases of pneumococcal meningitis occur each year. Before routine use of the pneumococcal conjugate vaccine, children younger than 1 year of age had the highest rates of pneumococcal

meningitis, approximately 10 cases per 100,000 population. The incidence of meningococcal meningitis is higher in sub-Saharan Africa (the so-called *meningitis belt*), extending from Senegal in the west to Ethiopia in the east. Although mortality rates have decreased as a result of improved medical management of CNS infections, in some children, developmental delay, learning disabilities, and behavioral problems may still occur. Thus CNS infections are a cause of immediate as well as long-term health concerns.

This chapter discusses the following:

- The interplay of host-related risk factors and virulence factors associated with the pathogens
- Characteristics of the CSF in CNS infections
- Microbial agents of CNS infections
- Laboratory diagnosis of CNS infections

General concepts related to infections of the central nervous system

Anatomic organization

The CNS encompasses the brain, spinal cord, and cranial nerves, but not the peripheral nerves (Fig. 35.1). The brain and spinal cord are protected by the skull and vertebral column, respectively, and three layers of meninges: the dura mater, arachnoid mater, and pia mater. The dura mater is a thick, fibrous, white membrane that is firmly adherent to the overlying skull. Below the dura mater is the arachnoid mater, followed by the pia mater.

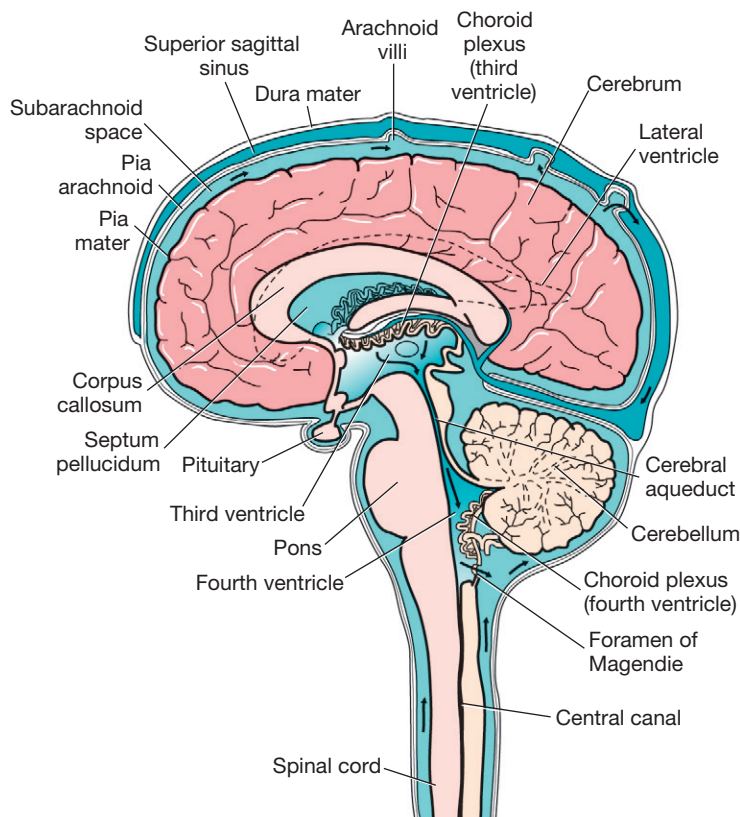


Fig. 35.1 Components of the central nervous system and flow pattern of cerebrospinal fluid.

The subarachnoid space between the arachnoid mater and pia mater is occupied by surface blood vessels and CSF. CSF is produced by filtration and secretion from specialized capillary tufts of the choroid plexus in the four ventricles of the brain. CSF flows from the two lateral ventricles to the third ventricle and enters the fourth ventricle via the aqueduct of Sylvius. From here, CSF enters the basal cisterns and circulates over the cerebellum and convexities of the cerebral hemispheres. CSF is absorbed primarily by the arachnoid villi through tight junctions of the endothelium.

Cerebrospinal fluid characteristics

CSF is a clear, colorless, and sterile fluid. In normal adults, CSF volume ranges from 90 to 150 mL, the protein concentration is 15 to 45 mg/dL, and the glucose concentration is 40 to 80 mg/dL (ratio of CSF glucose to serum glucose is 0.6). In normal adults, CSF contains 0 to 7 leukocytes per microliter, with a differential count of 60% to 80% lymphocytes, 10% to 40% monocytes, and 0% to 15% neutrophils. Compared with adults, normal newborns have higher CSF concentrations of protein (15 to 150 mg/dL), glucose (30 to 120 mg/dL), and leukocytes (0 to 30 per milliliter), with a greater percentage of monocytes and neutrophils. The paucity of leukocytes and protein (including immunoglobulins) in CSF provides little initial defense against invading organisms. Infections of the CNS are frequently, but not invariably, associated with an increase in CSF cell count (**pleocytosis**) and alterations in glucose and protein levels.

Host-pathogen relationships

Infection results from the complex interplay among the host, organism, and environment. Host risk factors that predispose to infection include extremes of age; nutritional and immunologic status; comorbidities, such as alcoholism, diabetes mellitus, malignancy, renal failure, and head trauma; and neurosurgical procedures. Structural components of the organism, such as capsule, pili, and fimbriae, which mediate adherence to respiratory tract epithelial cells, play an important role in meningeal infection. Additionally, the bacterial capsule can resist neutrophil phagocytosis and complement-mediated bactericidal activity, thus enhancing survival in the bloodstream. Host defenses include the presence of mucosal immunity mediated via immunoglobulin A (IgA) antibody, complement activation, and the presence of organism-specific antibodies. The CNS is normally a sterile site.

Central nervous system infections

Meningitis

Acute **meningitis** is commonly caused by bacteria (e.g., *S. pneumoniae*, *Neisseria meningitidis*, *H. influenzae*, *Listeria monocytogenes*) or viruses (e.g., enteroviruses [EVs], herpesvirus, mumps virus). Less commonly, it is caused by other organisms, such as fungi (e.g., *Cryptococcus neoformans* and *Cryptococcus gattii*), spirochetes (e.g., *Treponema pallidum*, *Borrelia burgdorferi*), protozoa (e.g., *Naegleria fowleri*), or helminths (e.g., *Angiostrongylus cantonensis*). Patients with acute meningitis usually have fever, headache, vomiting,

BOX 35.1 Bacteria involving meningitis related to age

Neonates

Streptococcus agalactiae (group B *Streptococcus*)
Streptococcus pneumoniae
Listeria monocytogenes
Escherichia coli

Babies and young children

Streptococcus pneumoniae
Neisseria meningitidis
Haemophilus influenzae
Streptococcus agalactiae (group B *Streptococcus*)
Mycobacterium tuberculosis

Teens and young adults

Neisseria meningitidis
Streptococcus pneumoniae

Older adults

Streptococcus pneumoniae
Neisseria meningitidis
Haemophilus influenzae
Streptococcus agalactiae (group B *Streptococcus*)
Listeria monocytogenes

From Centers for Disease Control and Prevention. (2021). *Meningitis: bacterial meningitis*. Available at: <https://www.cdc.gov/meningitis/bacterial.html>. (Accessed 17 June 2022).

photophobia, and altered mental status. In infants and children, irritability, restlessness, and poor feeding may be the only signs of meningitis. Untreated meningitis can result in obtundation, coma, and death.

Bacterial meningitis

The likely causative agents of bacterial meningitis depend on the age of the patient (Box 35.1) and on host factors, such as immune status, presence of a CSF leak, or presence of a foreign body, such as a ventriculoperitoneal shunt.

Pathogenesis

Bacterial infection of the leptomeningeal space, the space between the arachnoid membrane and pia mater, can occur from a distant focus via the bloodstream or by direct invasion from a contiguous focus of infection (e.g., mastoid or paranasal sinuses) or as a result of neurosurgical procedures. Most commonly, infection or colonization of the respiratory tract is followed by invasion of blood from a respiratory focus and seeding of the meninges. Once in CSF, bacteria replicate, release bacterial components, and cause an inflammatory reaction.

S. pneumoniae, a gram-positive diplococcus, is the most common cause of meningitis in adults and children (Fig. 35.2). Of more than 90 serotypes, only a few serotypes—including 4, 6B, 9V, 14, 18C, 19F, and 23F—accounted for most cases of invasive childhood pneumococcal infections in the United States before the implementation of routine immunization in infants with the heptavalent pneumococcal conjugate vaccine (PCV7). Serotype 19A has emerged as the most common cause of invasive disease and the serotype most associated

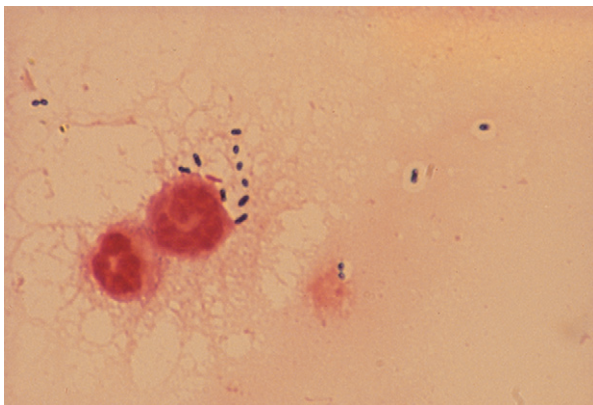


Fig. 35.2 Direct smear of acute bacterial meningitis in an adult showing the lancet-shaped, gram-positive diplococci characteristic of *Streptococcus pneumoniae*. The polysaccharide capsule produces a prominent halo around organisms (Gram stain, noncytocentrifuge preparation, $\times 1000$).

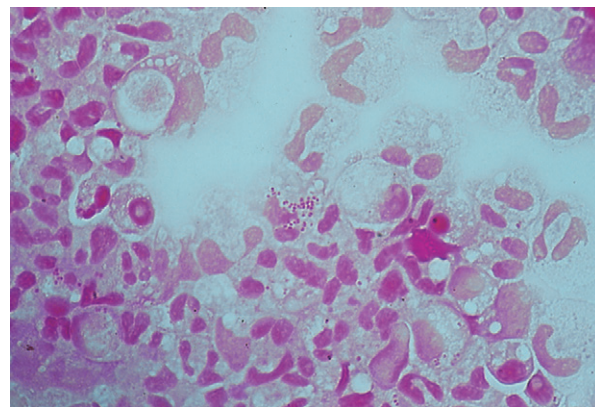


Fig. 35.3 Direct smear of the cerebrospinal fluid from a high school student showing clusters of gram-negative diplococci consistent with *Neisseria meningitidis* within polymorphonuclear leukocytes. Note the increased cellularity of the smear in this cytocentrifuge preparation (Gram stain, $\times 1000$).

with resistance in PCV7-immunized children. Patients with sickle cell anemia, those who have asplenia, and those with malignancy, malnutrition, and chronic renal or liver disease are more likely to develop serious pneumococcal disease.

Three pneumococcal conjugate vaccines are available for use in adults and children in the United States: (1) the 13-valent pneumococcal conjugate vaccine (PCV13), which is composed of purified polysaccharides of 13 serotypes conjugated to a nontoxic variant of diphtheria toxin carrier protein, CRM197; (2) the 15-valent conjugate vaccine (PCV15); and (3) the 20-valent conjugate vaccine (PCV20). A fourth vaccine is the 23-valent pneumococcal polysaccharide vaccine (PPSV23); it is composed of 23 purified capsular polysaccharides. The Centers for Disease Control and Prevention recommends PCV13 for children younger than 2 years of age.

H. influenzae type b, a gram-negative coccobacillus, is an important cause of bacterial meningitis in children. In addition to meningitis, it can cause otitis media, pneumonia, and epiglottitis. *H. influenzae* was previously the most common cause of bacterial meningitis, especially in young children. Most cases were caused by the capsular type b strains. Since the adoption of the routine use of conjugate vaccines against *H. influenzae* type b, there has been a marked reduction in the number of cases of *H. influenzae* meningitis. In developing countries with limited vaccine coverage, however, it continues to be an important cause of bacterial meningitis. In the United States, the proportion of invasive infections caused by strains of *H. influenzae* not type b have increased.

N. meningitidis, a gram-negative diplococcus (Fig. 35.3), is classified into 13 serogroups based on antigenically distinct, non-cross-reactive capsular polysaccharides. Serogroups A, B, C, X, Y, and W135 account for most cases of meningococcal disease throughout the world. Serogroups B, C, and Y account for most cases in Europe and the United States. Disease attributable to serogroup A is seen in Asia and Africa but is rare in industrialized countries. Disease caused by serogroups A and C can occur in epidemics. Outbreaks have also been reported with the W135 serogroup. Several clusters of serogroup B meningococcal disease have occurred on college campuses in the United States. Serogroup X causes a substantial number of cases of meningococcal disease in parts of Africa but is rare on other continents. Individuals who are deficient in terminal complement components (C5–C9) or properdin are

at a higher risk for meningococcal infections. In the United States, three quadrivalent conjugate meningococcal vaccines are licensed for use in children and adults against serogroups A, C, Y, or W, and two recombinant serogroup B are licensed for people 16 to 23 years of age.

L. monocytogenes is a gram-positive rod. Infection by this organism is usually seen in pregnant women, neonates, older adults, persons with alcoholism, and persons with impaired cell-mediated immunity. Outbreaks of *Listeria* infection have been associated with the consumption of contaminated coleslaw, milk, ice cream, and cheese. Patients with meningitis caused by *Listeria* have low CSF leukocyte counts, with a predominance of lymphocytes.

Streptococcus agalactiae (or GBS) is a gram-positive coccus that is often isolated from rectal or vaginal cultures of asymptomatic pregnant women. Early-onset GBS disease usually occurs within the first 24 hours of life and is not commonly associated with meningitis (5% to 10% of cases). Late-onset disease, which typically occurs at 3 to 4 weeks of age (range 7 to 89 days), commonly manifests as occult bacteremia or meningitis (approximately 30% of cases). Approximately 50% of survivors of early- or late-onset meningitis have long-term neurologic sequelae. Hospital transmission via the hands of health care workers has been described. Most cases of neonatal meningitis are caused by serotype III. Risk factors for GBS infection in adults include age older than 60 years, diabetes mellitus, and underlying malignancy.

Aerobic gram-negative bacilli, such as *Escherichia coli*, *Klebsiella* spp. (Fig. 35.4), *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Serratia* spp., and *Salmonella*, can also cause meningitis. In addition to neonates, older adults, patients with head trauma, or those who have undergone neurosurgical procedures are also at risk of meningitis caused by these organisms. Most strains of *E. coli* that cause meningitis possess the K1 antigen.

Other bacteria

Meningitis caused by *Staphylococcus aureus*, coagulase-negative staphylococci, or *Abiotrophia* and *Granulicatella* spp. usually occurs in patients who have undergone recent neurosurgical procedures or in those with CSF shunts. Meningitis caused by enterococci or group A streptococci are not commonly seen. Meningitis caused by anaerobic streptococci, *Bacteroides* spp.

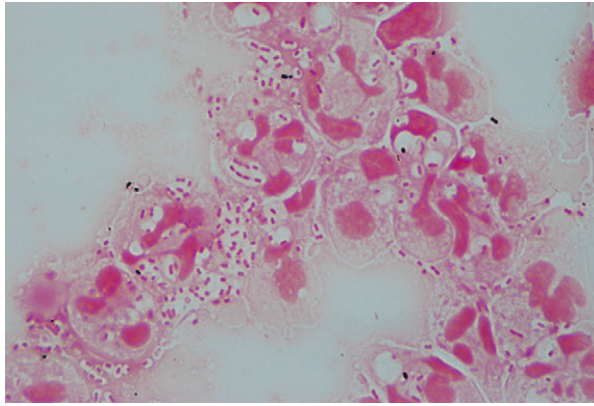


Fig. 35.4 Direct smear of posttraumatic, acute bacterial meningitis showing numerous intracellular and extracellular gram-negative bacilli with the prominent capsules characteristic of *Klebsiella pneumoniae* (Gram stain, cytocentrifuge preparation, $\times 1000$).

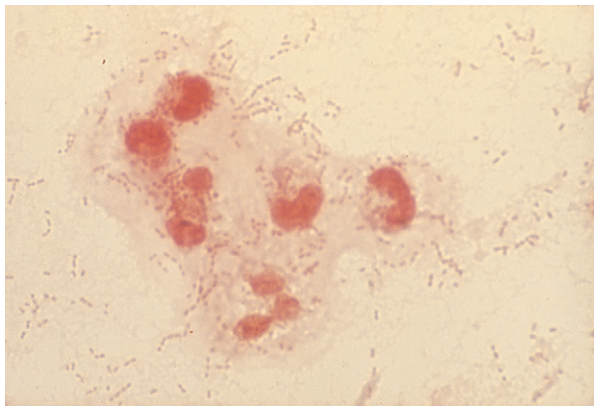


Fig. 35.5 Direct smear of cerebrospinal fluid from a newborn patient born to a woman with amnionitis secondary to premature rupture of the membranes. Numerous short, gram-negative bacilli in chains consistent with a *Bacteroides* sp. are seen. The organism could easily be confused with a *Streptococcus* sp. if the Gram stain were improperly decolorized (Gram stain, noncytocentrifuge preparation, $\times 1000$).

(Fig. 35.5), and *Fusobacterium* spp. is uncommon and is usually associated with a concurrent **brain abscess** or a contiguous focus of infection.

Shunt infections

Generally, CSF shunts are placed in patients with hydrocephalus or other CNS lesions that interfere with the flow of CSF. The proximal end of the shunt is in the cerebrospinal space, and the distal end is in the peritoneal, pleural, or vascular space. Patients with shunts are at risk of developing infections. Staphylococci account for two thirds of CSF shunt infections, with coagulase-negative staphylococci being the most common, followed by *S. aureus*. Gram-negative organisms, such as *E. coli*, *Klebsiella* spp., and *Proteus* spp., also can cause CSF shunt infections. An increasing incidence of CSF shunt infections from *Cutibacterium acnes* has been reported. Immunosuppressed patients can develop CSF shunt infections with *Candida* spp. CSF shunts terminating in the peritoneal cavity have a greater risk of infection with gram-negative organisms; mixed infections are seen when the catheter perforates a hollow viscus.

Viral infections

Case study

A 36-year-old male patient with human immunodeficiency virus (HIV) infection presented to the emergency department with complaints of inability to urinate for 3 days. He also reported numbness and weakness in his right leg for 7 months, a 25-lb weight loss, and fecal incontinence. Physical examination showed that he was afebrile, dehydrated, and emaciated, with bilateral lower extremity weakness and decreased deep tendon reflexes. Lesions of Kaposi sarcoma, thrush, and perianal herpetic vesicles were observed. Magnetic resonance imaging revealed no evidence of spinal cord compression, and a presumptive diagnosis of polyradiculopathy secondary to acquired immune deficiency syndrome (AIDS) was made. A lumbar puncture was performed. CSF showed an increased protein level (326 mg/dL) and a leukocyte count of 1720/mL, with 86% neutrophils.

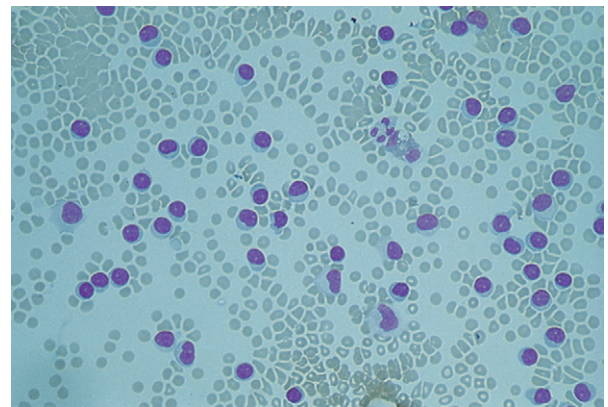


Fig. 35.6 Cytocentrifuge preparation of cerebrospinal fluid in a case of aseptic meningitis. Lymphocytes are present, and in this case the background is bloody. No organisms are seen (Wright stain, $\times 200$).

Viruses are the most frequent cause of **aseptic meningitis**, a condition characterized by lymphocytic pleocytosis in CSF and lack of an identifiable causative agent after routine stains and culture of CSF (Figs. 35.6 and 35.7). The most common viruses producing aseptic meningitis include EVs and herpesviruses. Less common causes of viral meningitis include mumps virus, lymphocytic choriomeningitis virus (LCMV), and HIV (Box 35.2). Viral pathogens colonize various mucosal surfaces in the body, such as the respiratory and gastrointestinal (GI) tracts. Some viruses (e.g., EVs, adenoviruses, and parvoviruses) can resist inactivation by gastric acid. After initial replication at the site of mucosal colonization, viremia develops, followed by invasion of the CNS. Early viral infections may show a predominance of neutrophils in CSF, but the pleocytosis rapidly progresses to a lymphocytosis.

Determination of a specific causative virus associated with a CNS infection is difficult because of the following: (1) a large number of different viruses involve the CNS; (2) viruses may come from endogenous reactivation or exogenous infection; (3) many viruses produce a spectrum of neurologic illnesses; and (4) the magnitude of any neurologic illness resulting from viruses varies based on the age and immune status of

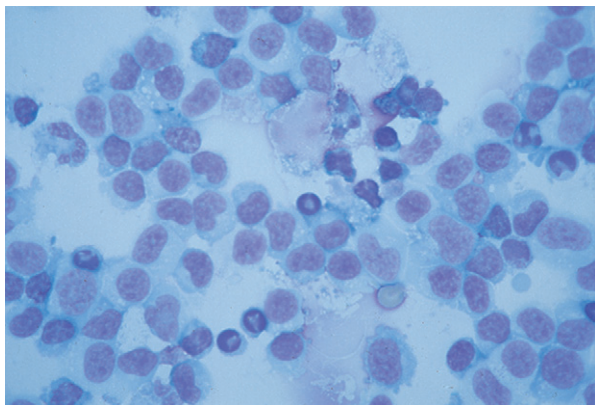


Fig. 35.7 Cytocentrifuge preparation of cerebrospinal fluid in meningitis resulting from tularemia. Reactive lymphocytes with monocytoid features are the only clue that this is not a viral infection. No organisms are seen. Cultures were positive (Wright stain, $\times 400$).

BOX 35.2 Viral agents involving the central nervous system

Enteroviruses

Coxsackieviruses A and B
Echoviruses
Parechoviruses
Polioviruses
Eastern equine encephalitis
Western equine encephalitis
Venezuelan equine encephalitis
St. Louis encephalitis virus
La Crosse virus
Colorado tick fever virus

Herpesviruses

Herpes simplex viruses 1 and 2
Epstein-Barr virus
Cytomegalovirus
Varicella-zoster virus

Others

Human immunodeficiency virus
Mumps virus
Dengue virus
Nipah virus
Rabies virus

the patient as well as other undefined factors. The determination of a specific viral cause can often be made with a careful patient history; selected serologic tests (e.g., determination of virus-specific immunoglobulin M [IgM] or of a fourfold or greater increase in antibody titer between acute-phase and convalescent-phase sera); a viral culture or polymerase chain reaction (PCR) assay for selected viruses; and tissue biopsy for routine light microscopy, immunofluorescence, or ultrastructural studies. Viruses isolated from body sites other than the CNS may be implicated in CNS syndromes. Appropriate specimens for culture include nasopharyngeal swabs, urine, stool, tissue, and occasionally blood.

Enteroviruses

EVs belong to the genus *Enterovirus* within the family Picornaviridae and include polioviruses, coxsackieviruses A and B, rhinoviruses, and echoviruses. EVs that are frequently associated with neurologic illness include coxsackievirus B and echoviruses. Although many different serotypes can cause meningitis, coxsackievirus serotypes A6, A9, B2, B3, B4, and B5; EV 71; and echovirus serotypes 4, 6, 9, 11, 16, 18, and 30 are the most common. In 2014, there were several reports of paralysis and neurologic illness in children caused by infection with EV D68.

In temperate climates, enteroviral infections are more common in summer, but in subtropical areas, no marked seasonality is observed. These infections are acquired via the fecal-oral route. Most cases of enteroviral meningitis are uncomplicated, with signs and symptoms resolving in 2 to 7 days. Most poliovirus infections are asymptomatic or cause aseptic meningitis, but a small proportion of infections progress to destruction of motor neurons in the anterior spinal cord, resulting in paralysis. Although the incidence of paralytic poliomyelitis has decreased dramatically since effective vaccines became available, outbreaks continue to occur in some parts of the world. A paralytic case was reported in 2022 in New York, the first case in the United States since 2013. The patient had no history of vaccination against poliovirus and was infected with a revertant or novel oral polio vaccine 2. A similar strain was isolated in London sewage also in 2022. Parechoviruses (PeVs) are members of the genus *Parechovirus* within the family Picornaviridae. PeV types 1 and 3 are associated with neonatal sepsis and meningitis.

Arboviruses

Arboviruses include a group of viruses transmitted by vectors, such as mosquitoes, ticks, sandflies, and other biting arthropods. Several of these viruses cause encephalitis, but aseptic meningitis and meningoencephalitis can also occur. The common arboviruses causing aseptic meningitis include the St. Louis encephalitis (SLE) virus, La Crosse (LAC) virus, Eastern equine encephalitis (EEE) virus, Western equine encephalitis (WEE) virus, West Nile virus (WNV), and Dengue virus (DENV). Neurologic manifestations of dengue infection (encephalitis, encephalopathy, occasionally meningitis) have been reported in multiple countries in infants, children, and adults. Japanese encephalitis virus, a mosquito-borne flavivirus, is endemic in parts of Asia. It can produce a severe encephalitis characterized by coma, seizures, paralysis, and abnormal movements. About one third of patients die, and serious sequelae are common in a significant proportion of survivors. In Mediterranean countries, Toscana virus, which belongs to the family Bunyaviridae, has also been identified as an important causative agent of acute meningitis and meningoencephalitis.

Mumps virus

The mumps virus, a member of the family Paramyxoviridae, commonly causes parotitis (infection or inflammation of the parotid salivary glands). Aseptic meningitis is the most common neurologic complication. Symptoms of meningitis occur in up to 30% of patients with mumps parotitis within 4 to 10 days of illness. Occasionally, meningeal symptoms can precede parotitis by up to 7 days. Infection of the CNS is usually self-limiting and associated with complete recovery. Due to routine vaccination, mumps and its complications

are rare in developed countries. Mumps virus vaccine is an attenuated vaccine that is usually given as part of the combined measles-mumps-rubella vaccine.

Herpesvirus

Herpesviruses are DNA viruses. They include herpes simplex virus (HSV)-1, HSV-2, Epstein-Barr virus (EBV), cytomegalovirus (CMV), varicella-zoster virus (VZV), human herpesvirus (HHV)-6, HHV-7, and HHV-8. Herpesviruses account for 0.5% to 3% of all cases of aseptic meningitis. Although aseptic meningitis can occur with any of these viruses, only meningitis associated with HSV is common. The clinical outcome in patients with aseptic meningitis resulting from herpesvirus is indistinguishable from that of other causes, and the outcome is uniformly good. It is important, however, to differentiate between HSV encephalitis, a potentially fatal disease, and the self-limiting HSV aseptic meningitis. Infants born to mothers with genital herpes are at risk for serious infection. HSV is associated with Mollaret's meningitis, also called recurrent lymphocytic meningitis, a recurring form of meningitis.

Influenza

Influenza viruses are members of the family Orthomyxoviridae. In children younger than 5 years of age, CNS involvement (mainly presenting as encephalitis) is associated with influenza A virus and less frequently with influenza B virus.

Human immunodeficiency virus

HIV is a member of the family Retroviridae. Meningitis associated with HIV infection may occur as part of the primary infection or may occur later in the course of the infection. Patients with acute infection usually have aseptic meningitis as part of the mononucleosis-like syndrome. Patients presenting with chronic meningitis often have other associated symptoms, such as cranial neuropathies. In addition, patients with HIV can present with CNS involvement attributable to opportunistic pathogens.

Mycobacterial infections

Although uncommon, the most common mycobacterial infection of the CNS is tuberculous meningitis caused by *Mycobacterium tuberculosis*. Other mycobacteria associated with CNS infections include *Mycobacterium bovis*, *Mycobacterium avium*, *Mycobacterium intracellulare*, *Mycobacterium kansasii*, *Mycobacterium fortuitum*, *Mycobacterium abscessus*, and *Mycobacterium africanus*. Mycobacteria are acid-fast bacilli with a thick cell wall containing lipids, peptidoglycans, and arabinomannans. The bacilli enter the body through respiratory droplets and multiply in the alveolar spaces or macrophages. Spread to extrapulmonary sites occurs via the blood stream.

HIV infection is a risk factor for tuberculous meningitis. **Tuberculous meningitis** results when the brain tubercle ruptures into the subarachnoid space. Both the meninges and the brain itself are frequently involved, with a resulting thick exudate, especially at the base of the brain. The clinical presentation of tuberculous meningitis is subacute and includes fever, headache, **meningismus**, and mental changes. Meningismus, also called meningism, is a set of symptoms caused by non-meningitis irritation of the meninges usually associated with acute febrile illness. It is typically characterized by neck stiffness, photophobia, and headache. Vomiting and other signs of increased intracranial pressure may occur.

Spirochetal infections

The two spirochetes associated with CNS infection are *T. pallidum* subsp. *pallidum* and *B. burgdorferi*. *T. pallidum* subsp. *pallidum*, the causative agent of syphilis, enters the CNS during early infection and can be isolated from CSF in patients with primary syphilis. Many cases of neurosyphilis are reported in patients with HIV infection. Manifestations of neurosyphilis can occur at any stage of infection, especially in patients with HIV infection. Syphilitic involvement of the CNS can take one of four forms: syphilitic meningitis, meningovascular syphilis, parenchymatous neurosyphilis, and gummatous neurosyphilis.

Involvement of the CNS can occur in patients with Lyme borreliosis. It is usually seen in patients with early disseminated disease and is less likely during late disease. Not all cases of Lyme meningitis are preceded by the characteristic erythema migrans rash. In addition to signs of meningeal irritation, some patients with Lyme meningitis can have other neurologic manifestations of Lyme disease, such as cranial nerve neuropathy (commonly cranial nerve VII) and radiculoneuritis.

Fungal Infections

Case study

A 52-year-old male patient arrived at an emergency department in a disoriented and poorly responsive state and with labored breathing. The patient's history included poorly controlled diabetes and chronic obstructive pulmonary disease secondary to cigarette smoking. Current medications included oral steroids for pulmonary disease. On physical examination, the patient was febrile, lethargic, and in respiratory failure. A lumbar puncture was performed. Direct smear of the CSF using calcofluor white reagent showed encapsulated budding yeasts. Despite aggressive therapy with amphotericin B and 5-flucytosine, the patient's condition failed to improve, and he died on the third day of hospitalization.

Fungi are uncommon causes of CNS infections ([Box 35.3](#)). The risk factors for CNS fungal infections include immunocompromised state, organ transplantation, and diabetes. *Aspergillus* and *Cryptococcus* species are the most common etiologic agents in immunosuppressed patients. Other etiologic agents include *Coccidioides immitis*, *Histoplasma capsulatum*, and *Blastomyces dermatitidis*. Concurrent infections with *C. neoformans* and *H. capsulatum* have been reported in immunocompromised persons. Recent outbreaks caused by *Cryptococcus gattii* have been reported in the Pacific Northwest of the United States in previously healthy individuals. Rare cases of meningeal sporotrichosis caused by *Sporothrix schenckii* have been reported. The clinical presentation is usually of chronic meningitis. The white blood cell count is usually moderately elevated, with a predominance of lymphocytes. A predominance of eosinophils may occur in infections caused by *Coccidioides* organisms.

An outbreak of fungal meningitis and other infections was reported among patients who received injections of contaminated preservative-free methylprednisolone acetate solution. In addition to fungal meningitis, localized spinal or paraspinal infections, such as epidural abscess and arachnoiditis, and infections associated with injections in joints, were reported. The predominant fungus identified in patients was *Exserohilum rostratum*. One patient, the index case, had a laboratory-confirmed *Aspergillus fumigatus* infection.

BOX 35.3 Fungal organisms involving the central nervous system

Common

Cryptococcus neoformans
Coccidioides immitis

Uncommon

Histoplasma capsulatum
Candida spp.
Cryptococcus gattii
Aspergillus spp.
Blastomyces dermatitidis

Rare

Paracoccidioides brasiliensis
Pseudallescheria boydii
Mucorales (*Mucor*, *Rhizopus*, *Absidia*, and *Cunninghamella* spp.)
Sporothrix schenckii
Trichosporon beigelii
Penicillium spp.
Fusarium spp.
Alternaria spp.
Curvularia spp.
Acremonium spp.
Fonsecaea spp.
Bipolaris spp.
Drechslera biseptata
Exserohilum rostratum

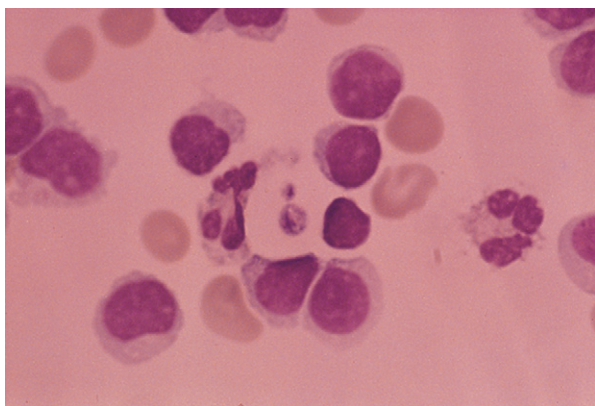


Fig. 35.8 Cytocentrifuge preparation of cerebrospinal fluid showing a single yeast with narrow-based budding and a prominent surrounding capsule characteristic of *Cryptococcus neoformans*. Cryptococcal meningitis in partially immunocompetent hosts may show only rare organisms mixed with an inflammatory background of lymphocytes, monocytes, and eosinophils (Wright stain, $\times 1000$).

C. neoformans is an encapsulated basidiomycetous yeast. It can spread hematogenously to the CNS from pulmonary foci and cause chronic meningitis, particularly in patients with AIDS. There are two varieties of *C. neoformans*: *C. neoformans* var. *neoformans* and *C. gattii* (formerly known as *C. neoformans* var. *gattii*). *C. neoformans* var. *neoformans* is the major isolate in patients with AIDS. *C. gattii* human infections have been reported in North America, South America, and Australia. In the United States, most cases have been reported in the Pacific Northwest and California. The number of organisms in the CSF may be small in immunocompetent patients (Fig. 35.8) but large in immunosuppressed patients (Fig. 35.9).

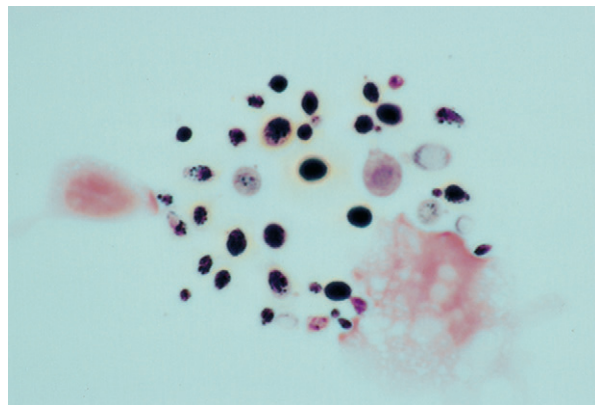


Fig. 35.9 Cryptococcal meningitis in immunosuppressed hosts may show numerous organisms and scarce or absent inflammation. Note the variation in size, variable Gram staining, and narrow-based budding. The organisms are evenly spaced because of their abundant polysaccharide capsules (Gram stain, cytocentrifuge preparation, $\times 400$).

C. immitis and *B. dermatitidis* are dimorphic fungi that can cause chronic meningitis. Inhalation of conidia results in pulmonary infection, which may spread to the CNS via the blood stream and cause an abscess or fulminant meningitis. Infections caused by *C. immitis* are limited to endemic regions, mainly the southwestern United States, Mexico, and Central and South America. *B. dermatitidis* is endemic in the Mississippi and Ohio river basins. In rare cases of disseminated histoplasmosis (*Histoplasma capsulatum*), involvement of the CNS is observed. Like *B. dermatitidis*, it is endemic in the Mississippi and Ohio river basins.

Candida spp. are a cause of fungal meningitis and cerebral abscesses. They are mainly seen in patients with invasive or disseminated candidiasis. Candidiasis may be acquired as a healthcare-associated infection in patients with indwelling catheters or those receiving prolonged antimicrobial therapy. CNS infection with *Candida* spp. can occur in patients with ventriculoperitoneal shunts. Patients with low peripheral blood neutrophil counts secondary to chemotherapy are at risk for candida infections. *Candida albicans*, *Candida tropicalis*, and *Candida parapsilosis* are the most commonly identified species.

Parasitic infections

Although uncommon, protozoa and helminths can invade the CNS and cause meningitis. Some parasites cause CNS lesions without obvious meningeal inflammation. They are discussed briefly in this section.

Protozoa

The free-living amebae that can infect humans include *N. fowleri*, *Acanthamoeba* spp., and *Balamuthia mandrillaris*. Trophozoites invade the nasal epithelium and migrate to the CNS via the olfactory nerve. *N. fowleri* can cause a rapidly progressive and almost always fatal **primary amebic meningoencephalitis**. *Acanthamoeba* spp. and *B. mandrillaris* usually cause granulomatous amebic encephalitis with a more insidious onset. *N. fowleri* is found in warm freshwater and moist soil. Most cases of infection have been associated with swimming in warm natural bodies of water. *Acanthamoeba* spp. are found in soil, freshwater, brackish water, and

sewage. No environmental sources have been identified for *B. mandrillaris*.

Cerebral malaria is an acute illness that occurs because of sequestration of the parasite *Plasmodium falciparum* in the CNS. It is characterized by changes in mental status, seizures, motor deficits, and coma. Human infection is initiated when the sporozoite stage of *P. falciparum* is injected into the bloodstream during mosquito feeding. Parasitized red blood cells develop knobs with cytoadherent surface properties, causing them to adhere to the endothelium of capillaries and venules in the brain.

Toxoplasma gondii is a coccidian, obligate, intracellular protozoan. Humans acquire infection by eating raw or undercooked meat containing tissue cysts or by contact with oocysts from feral or domestic cats. Organ transplant recipients may acquire toxoplasmosis from a donated organ. The seroprevalence of toxoplasmosis is higher in Europe and South America than in the United States. Sporozoites released from the ingested oocyst or tissue cysts invade the human small intestine, spread hematogenously, and invade cells of the viscera and possibly the brain. In immunocompromised patients, toxoplasmosis may result from primary infection or reactivation of a latent infection and commonly affects the CNS. CSF findings (Fig. 35.10) are nonspecific and include a mild lymphocytic pleocytosis and increased CSF protein level. The diagnosis of toxoplasmosis is usually serologic, although immunocompromised patients may not demonstrate a humoral immune response to the infection. In such cases, especially if there is a focal brain lesion, diagnosis can be obtained by brain biopsy. In patients with AIDS, the brain lesions have characteristic radiologic features; the diagnosis is often confirmed by clinical response to specific therapy.

Trypanosomes that infect humans include *Trypanosoma brucei* subsp. *gambiense*, *T. brucei* subsp. *rhodesiense*, and *Trypanosoma cruzi*. The first two are found predominantly in Africa. Humans are infected by the bite of tsetse flies. Infection with *T. brucei gambiense* results in a chronic meningoencephalitis more commonly known as *sleeping sickness*. CNS infection with *T. brucei rhodesiense* results in a more acute disease, often resulting in death. *T. cruzi*, the causative agent of Chagas disease, is found in Central and South America. In immunosuppressed patients, encephalitis caused by *T. cruzi* is observed.

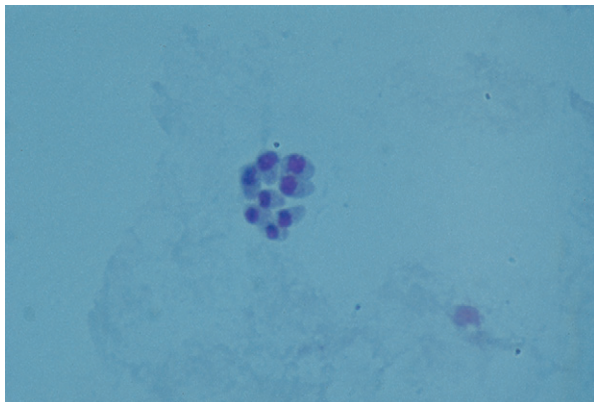


Fig. 35.10 Touch preparation of brain tissue showing typical floret of *Toxoplasma gondii* trophozoites. The organisms are not typically seen in cerebrospinal fluid preparations (Wright stain, $\times 1000$).

Helminths

Infection by the larval forms of the nematode *A. cantonensis* can cause eosinophilic meningitis. *A. cantonensis* infection is fairly common in certain parts of the world (e.g., Thailand, Malaysia, Vietnam). Humans become infected when they ingest larvae in snails or slugs or by eating green leafy vegetables contaminated by these parasites. In the CNS, the larvae usually do not complete their life cycle and eventually die, surrounded by an eosinophilic infiltrate. Both peripheral and CSF eosinophilia are usually seen in these patients.

Gnathostoma spinigerum, a GI tract parasite of wild and domestic dogs and cats, may cause eosinophilic meningoencephalitis in humans. *G. spinigerum* is common in Southeast Asia, China, and Japan. Humans acquire the infection following the ingestion of undercooked infected fish and poultry. *Baylisascaris procyonis* is an ascarid parasite prevalent in the raccoon population in the United States and has emerged as a causative agent of human eosinophilic meningoencephalitis. Human infections occur following ingestion of food products contaminated with raccoon feces.

Neurocysticercosis is a major cause of brain lesions in certain parts of the world. It is caused by the larvae of the pig tapeworm *Taenia solium*. Humans become infected by ingestion of food or water contaminated with eggs of *T. solium*. Larvae released from the eggs migrate through the intestinal wall to the CNS and form cysts in the subarachnoid space. CSF may be normal or may show pleocytosis, with a predominance of neutrophils or eosinophils and decreased glucose level. The diagnosis is confirmed by radiographic studies and demonstration of cyst antigen in CSF or serum using an enzyme-linked immunoelectron transfer blot test.

Echinococcus granulosus, a tapeworm, causes the formation of hydatid cysts in humans. Humans are infected during contact with intermediate hosts for the parasite, such as sheep and dogs. Although the liver and lungs are the most commonly affected organs, the brain may be invaded by the parasite, resulting in seizures. Human visceral larva migrans results from ingestion of eggs of *Toxocara canis* or *Toxocara cati*. Although most of the larval stages are seen in the liver, some larvae reach the CNS and cause lesions in the brain.

Paragonimus westermani, the oriental lung fluke, is known to cause brain lesions. Human infection is confined to Japan, South Korea, Thailand, China, and the Philippines. Infection occurs by ingestion of raw or improperly cooked crustaceans. Neurologic symptoms, such as epilepsy and paralysis, are observed when the brain is infected. Serologic assays may be useful for the detection of *P. westermani*.

Neuroschistosomiasis can occur when schistosomes migrate to the brain or spinal cord and deposit eggs. Myelopathy is seen in infection with *Schistosoma mansoni* and *Schistosoma haematobium*; acute encephalitis has been reported with *Schistosoma japonicum* infections.

Case check 35.1

Laboratory findings in CSF can aid the diagnosis of meningitis. CSF characteristics differ, depending on the cause. In general, cell types, concentrations of glucose and protein, staining characteristics of the organism, culture, and other testing provide clues to the causative agent.

Meningoencephalitis and encephalitis

Patients with **meningoencephalitis** or **encephalitis** have involvement of the cerebral cortex. The diagnosis of encephalitis is usually inferred from the clinical presentation. Because of diffuse involvement of the cerebral cortex in patients with encephalitis, mental status changes and other focal or diffuse neurologic signs, such as seizures, are common. The most common causes of encephalitis are viruses, including herpesviruses, EVs, and arboviruses. Acute meningoencephalitis caused by paramyxoviruses, such as Nipah virus, have been reported in abattoir workers. Fruit bats are reservoirs of Nipah virus, and outbreaks caused by drinking raw date palm sap contaminated by bat saliva and excreta have been reported in Asia. In 2018, a Nipah virus outbreak occurred in India with 91% mortality, and the source was identified as the fruit bat in the index case. Nonviral causes include *L. monocytogenes*, *Rickettsia*, *Bartonella*, *Mycoplasma*, *B. burgdorferi*, *N. fowleri*, and *T. gondii*.

Clues to the cause of the encephalitis are sometimes available on physical examination, such as the rashes of Lyme borreliosis and Rocky Mountain spotted fever (RMSF). A history of tick bite may suggest a diagnosis of RMSF, Colorado tick fever, Lyme borreliosis, or ehrlichiosis. Herpes simplex encephalitis in adults may follow primary herpesvirus infection or result from reactivation of a previous herpesvirus infection. Neonatal herpes simplex meningoencephalitis usually reflects disseminated herpetic disease. Beyond the neonatal period, HSV encephalitis is usually caused by HSV-1. Patients usually have fever, altered sensorium, and focal neurologic signs consistent with temporal lobe involvement. Encephalitis from other herpesviruses is less common.

Arboviruses are RNA viruses that demonstrate strong tropism for the CNS. These viruses are transmitted to humans by mosquitoes, ticks, or sandflies. Important arboviruses in the Western Hemisphere include members of the genus *Alphavirus* (EEE, WEE, and Venezuelan equine encephalitis [VEE] viruses), *Flavivirus* (SLE virus), *Orthobunyavirus* (LAC virus), and *Coltivirus* (Colorado tick fever virus). The incidence of these infections can differ with the geographic region. EEE, the most severe arthropod-borne encephalitis in the United States, is typically a fulminant disease leading to coma and death in one third of cases and serious neurologic sequelae in another third. It is endemic along the entire East Coast of the United States. Infections are most common in young children and older adults.

WEE occurs predominantly in the Midwestern and Western United States. The clinical severity of WEE is intermediate, with a case fatality rate of 5%. VEE is endemic in Central America and Florida. WEE and VEE are difficult to distinguish from EEE on clinical grounds, and infants and young children are at greatest risk for infection. Fortunately, the risk of fatal encephalitis is much lower (about 10% in WEE and 0.6% in VEE).

Despite its name, SLE has been reported throughout the United States. Children and older adults experience more severe illness. It can present with confusion, fever, slow disease progression, lack of focal neurologic findings, generalized weakness, and tremors. The risk of fatal encephalitis is estimated at about 10%. LAC virus is the most commonly isolated member of the California serogroup of bunyaviruses. Encephalitis resulting from LAC virus usually occurs in

Ohio, Illinois, Wisconsin, and Minnesota. Children are most commonly afflicted. Infections with LAC virus and the other California serogroup viruses are relatively benign arboviral infections, with a mortality rate of less than 1%.

WNV is a mosquito-borne flavivirus that commonly causes a self-limiting febrile illness, often associated with a rash. In 1999, the virus was introduced into the United States and spread rapidly. Neurologic involvement includes aseptic meningitis, myelitis, and fatal encephalitis. The mortality rate is about 5%; death occurs mainly in older adult patients. Colorado tick fever virus belongs to the genus *Coltivirus* of the family Reoviridae. In addition to meningoencephalitis, Colorado tick fever virus shows a peculiar tropism for bone marrow, and infection is often accompanied by leukopenia and thrombocytopenia.

Rabies is a zoonosis caused by an RNA virus of the genus *Lyssavirus*. Infection occurs through bites by infected animals, such as skunks, raccoons, bats, foxes, and unimmunized domesticated animals, such as dogs and cats. Initial symptoms include fatigue, GI symptoms, and pain at the bite wound. Rabies can present as acute encephalitis indistinguishable from other viral encephalitides or with the classic syndrome of agitation, emotional lability, seizures, and hallucinations. Diagnosis is confirmed with demonstration of the rabies antigen (by immunofluorescence) in neck skin biopsies or by demonstration of the rabies antigen in the neurons of the brains of patients or infected animals.

More recently, associations between Zika virus (single-stranded RNA arbovirus member of the genus *Flavivirus*) and congenital CNS malformation have been reported. Cases of Zika virus associated with meningoencephalitis in adults were diagnosed by culture and PCR of CSF specimens.

Brain abscesses

Brain abscesses are circumscribed areas of tissue destruction containing organisms and inflammatory cells. A cerebral abscess begins as a focal area of acute inflammation, followed by the development of a necrotic center and the presence of macrophages and fibroblasts in the periphery. Eventually, there is a diminution in the necrotic center and formation of a collagenous capsule.

Most cerebral abscesses occur as a result of spread from a contiguous focus of infection in the middle ear, mastoid cells, or paranasal sinuses. Brain abscesses secondary to ear infections are usually localized in the temporal lobe or the cerebellum, and those attributable to spread from paranasal sinuses or from dental infections are seen in the frontal lobe. The second common mechanism for development of brain abscess is by hematogenous spread from a distant focus of infection, such as lung abscess, infective endocarditis, and intraabdominal infections.

Brain abscesses can also develop secondary to trauma with dural breach or following neurosurgery. In some patients, none of these pathogenic mechanisms are evident. The microorganism isolated from a brain abscess often depends on the predisposing condition. In patients with brain abscess secondary to ear or sinus infection, the common organisms include streptococci, *Bacteroides* spp., and *Prevotella* spp., whereas in patients with penetrating trauma or infective endocarditis, *S. aureus* is more likely to be identified. Neutropenic or transplant patients are more susceptible to developing infections

by fungi, such as *Aspergillus* or *Mucorales* spp.; patients with HIV are more likely to have infections by *T. gondii*, *Nocardia* spp., or *Mycobacterium* spp.

Bacterial pathogens

Streptococci are the most common organisms isolated from nontraumatic brain abscesses (Fig. 35.11). Streptococci, such as *Streptococcus anginosus*, *Streptococcus intermedius*, and *Streptococcus constellatus*, are isolated in 50% to 70% of cases. Mixed infections are seen in 30% to 40% of cases. Anaerobes commonly identified from brain abscess include *Bacteroides* spp. and *Prevotella* spp. Members of the order Enterobacterales, such as *E. coli*, *Proteus* spp., and *Enterobacter* spp., can sometimes cause brain abscesses. *Citrobacter diversus* meningitis is often associated with brain abscesses. In neonates, *Cronobacter sakazakii* has been reported to cause brain abscesses. *Actinomyces* and *Nocardia* spp. may also be isolated from patients with brain abscesses. Nocardial abscesses are more common in patients with HIV, organ transplant recipients, and those receiving corticosteroid therapy. It is important that samples obtained at the time of surgery be cultured under aerobic and anaerobic conditions.

Fungal pathogens

The increasing use of immunosuppressive drugs and broad-spectrum antimicrobials has contributed to the increasing incidence of fungal brain abscesses. In addition to *Candida* spp., *Aspergillus*, *Mucor*, *Rhizopus*, *Absidia*, and pigmented fungi (*Cladosporium trichoides*, *Xylohypha bantiana*, *Cladosporium bantianum*, *Cladophialophora bantiana*) are common fungi that can cause brain abscesses. Infection caused by *Candida* spp. is more common in patients with indwelling catheters, those receiving hyperalimentation, or those receiving long-term steroid therapy. *Aspergillus* infection and cerebral phaeohyphomycosis result from dissemination of the organism from a primary focus, usually the lung, or by direct extension, such as from paranasal sinuses. Presence of pigmented fungi in aspirates or tissue specimens is diagnostic for phaeohyphomycosis. Mucormycosis is more commonly seen in patients with diabetic ketoacidosis or hematologic malignancies or following transplants. The organism enters

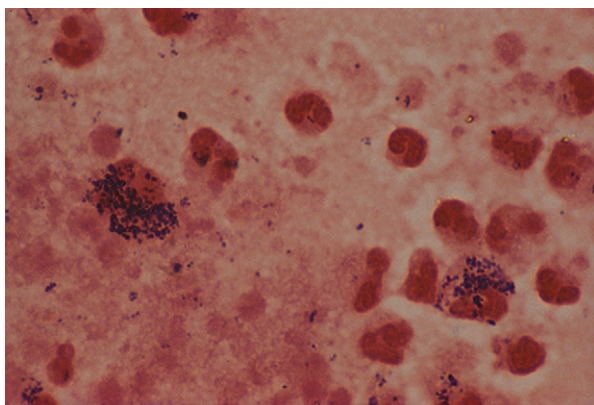


Fig. 35.11 Direct smear of aspirated brain abscess contents. Clusters of intracellular gram-positive cocci in groups are consistent with microaerophilic streptococci (Gram stain, noncytacentrifuge preparation, $\times 1000$).

the CNS from paranasal sinuses by eroding the frontal bone to reach the CNS (rhinocerebral mucormycosis) or by hematogenous dissemination. Other fungi occasionally isolated from cerebral abscesses include *B. dermatitidis*, *C. neoformans*, *Coccidioides* spp., and *Pseudallescheria boydii*.

Case check 35.2

In CNS infections, such as brain abscesses, in which meningeal involvement is limited, CSF findings are not very characteristic and hence cannot be relied on to make a diagnosis. Accurate diagnosis of the causative agent may depend on tissue samples or aspirates or on serologic tests.

In the current coronavirus disease 2019 (COVID-19) pandemic caused by the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), besides acute respiratory involvement, CNS manifestations (dizziness, headache, impaired consciousness, acute cerebrovascular disease, ataxia, epilepsy, nerve pain, and a reduction in taste, smell, and visual perception) were also reported. SARS-CoV-2, an RNA virus that belongs to the family Coronaviridae, was identified in neural and capillary endothelial cells in the frontal lobe of autopsied patients who died of COVID-19. The neurons and glial cells express the angiotensin-converting enzyme 2 (ACE2) receptor, the target for SARS-CoV-2. The neurologic manifestations may be caused by neuroinvasion or immune hyperactivation. Some patients develop the autoimmune Guillain-Barré syndrome. In general, patients presenting with symptoms of respiratory and/or CNS involvement caused by SARS-CoV-2 are diagnosed by a reverse-transcription polymerase chain reaction (RT-PCR) or antigen test using nasopharyngeal or nasal swabs.

Laboratory diagnosis

The diagnosis of CNS infections is based on examination of CSF, aspirates, and tissue samples. Blood cultures are often helpful in identifying the causative microorganism. A lumbar puncture is performed by inserting a sterile hollow needle into the spinal subarachnoid space in the lower (lumbar) back (Fig. 35.12). Lumbar puncture is often performed after computed tomography (CT) in patients with elevated intracranial pressure or focal neurologic lesions because of the risk of brain herniation. In patients with a brain abscess, aspirates and tissue samples are helpful because CSF may be normal.

Cerebrospinal fluid transport and analysis

CSF samples should be transported to the laboratory without delay and processed as soon as possible to prevent loss of viability of the causative agent. If delay cannot be avoided, CSF samples should be stored at room temperature until processed (within 24 hours). For viral testing, CSF samples may be stored at 2°C to 8°C in the short term (<48 hours) and at -70°C in the long term. When large volumes (>1 mL) of CSF sample are available, concentration by centrifugation increases the yield of microorganism for microscopic examination and culture. It is standard to obtain three to four tubes of CSF, each containing 1 or 2 mL of fluid. Cultures are

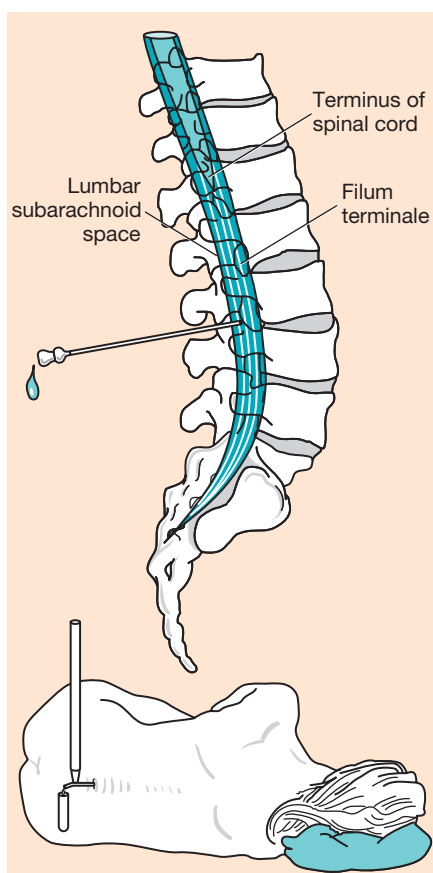


Fig. 35.12 Technique of lumbar puncture. The cerebrospinal fluid sample is obtained by inserting a long, sterile, hollow needle into the spinal subarachnoid space in the lumbar region.

performed on samples that have the least likelihood of contamination (second or third tube).

The CSF is analyzed for glucose and protein concentration, cell counts, and identification of the causative agent by Gram stain, culture, antigen detection, and PCR assay. Meningitis is suspected from the presenting clinical symptoms, findings on physical examination, and initial CSF studies, including visual inspection, chemical analysis, and cell counts. The characteristic CSF laboratory findings for various causative agents are compared in Table 35.1. The CSF white blood cell counts in neonates may not be helpful in the diagnosis of bacterial meningitis. Acid-fast staining and negative staining are useful in the diagnosis of tuberculous and cryptococcal meningitis, respectively. Cryptococcal antigen detection based on latex agglutination, enzyme immunoassay, and later flow methods are commercially available. Blood cultures are often positive in patients with bacterial meningitis if obtained before administration of antimicrobial therapy.

Culture

Isolation of fastidious organisms require special medium and incubation conditions. Anaerobic bacteria rarely cause meningitis but are commonly associated with brain abscesses. Transport media, such as the modified Stuart medium or Amies medium, are generally sufficient for transport of specimens. Sheep blood and chocolate agar plates incubated in 3% to 5% carbon dioxide (CO₂) are usually used for bacterial culture of CSF. Anaerobic cultures and prolonged incubation of broth media have been found to enhance the recovery of *Cutibacterium* spp. and *Propionibacterium* spp. from CSF cultures. When CSF samples are collected from shunts, broth media should also be inoculated. Blood cultures are not

Table 35.1 Characteristic cerebrospinal fluid findings in meningitis

Parameter	Organism					
	Bacterial	Fungal	Tuberculous	Syphilitic	Viral	Parasitic
Organisms seen in CSF	Usually	Less common	Rare	None	None	Rare
Cell count (leukocytes/ μ L)	100–100,000, neutrophils predominate	Normal– 500, lymphocytes predominate ^a	50–500, lymphocytes predominate ^b	100–750, lymphocytes predominate	Normal– 200, lymphocytes predominate ^c	Normal– 200, lymphocytes and/or eosinophils predominate
Protein ^d (mg/dL)	100–500	Normal–250	Normal–150	50–250	Frequently normal	Usually increased
Glucose ^e	Usually markedly decreased	Normal to decreased	Usually decreased	Normal	Normal	Normal to decreased
Additional findings	Bacterial antigen test	Calcofluor-concentrated specimen; latex agglutination (e.g., <i>Cryptococcus neoformans</i>)	PCR, auramine-rhodamine on concentrated specimen	Positive VDRL test result	Serology, PCR, culture, biopsy	Serology, biopsy

CSF, Cerebrospinal fluid; PCR, polymerase chain reaction; VDRL, Venereal Disease Research Laboratory.

NOTE: Critical values—any positive result of a microbiological test, such as direct stained smear, culture, polymerase chain reaction, or antigen testing, is considered critical and should be reported to the health care provider immediately.

^aMay have normal CSF cell count with *C. neoformans*. Eosinophils may predominate in *Coccidioides immitis* infection.

^bNeutrophils may predominate in early meningitis.

^cCSF cell count may be more than 1000 leukocytes per milliliter in lymphocytic choriomeningitis virus infection. Neutrophils may predominate in early meningitis.

^dCSF protein levels are age and laboratory dependent. A typical reference range for adults is 20 to 60 mg/dL.

^eCSF glucose levels are typically 60% of the blood glucose level. The reference range for the CSF/plasma ratio is 0.3 to 0.9.

useful in most CNS device infections but could be useful in ventriculoatrial shunt infections.

Molecular diagnostics

The BioFire FilmArray meningitis/encephalitis (ME) panel (biomérieux, Durham, NC) is a multiplexed in vitro diagnostic test that can be used to detect 14 pathogens (*E. coli* K1, *H. influenzae*, *L. monocytogenes*, *N. meningitidis*, *S. pneumoniae*, *S. agalactiae*, CMV, EV, HSV-1, HSV-2, HHV-6, PeV, VZV, and *C. neoformans*/*C. gattii*) simultaneously in CSF samples. The panel allows rapid detection (1 hour) with good sensitivity and specificity. A negative result in CSF samples collected from intrathecal devices should be interpreted with caution. Gram stain and culture will still need to be performed to identify pathogens not covered by the panel. Commercial assays such as the Xpert EV assay (Cepheid, Sunnyvale, CA), NucliSENS EasyQ Enterovirus assay (bioMérieux), and Simplexa HSV 1 & 2 Direct assay (DiaSorin, Cypress, CA) are some additional nucleic acid based assays that are cleared by the U.S. Food and Drug Administration (FDA) for the detection of viruses in CSF specimens. A list of FDA-cleared nucleic acid-based diagnostic tests can be found at <https://www.fda.gov/medical-devices/in-vitro-diagnostics/nucleic-acid-based-tests#microbial>.

Next-generation sequencing (NGS), still primarily a research method, can aid in the identification of viral agents of CNS infections, especially when traditional diagnostic methods fail. Novel cyclovirus, gemicircularvirus, astrovirus, and coronavirus OC43 have been identified in CSF specimens of patients with CNS infections of unknown cause by using NGS.

Bacterial infections

In acute bacterial meningitis, CSF is turbid or cloudy because protein levels are significantly increased. Glucose levels are low (<40% of the serum glucose concentration) in most patients with bacterial meningitis. The gold standard for diagnosis of bacterial meningitis is culture of CSF. Antimicrobial therapy reduces culture sensitivity. Staining techniques are rapid but less sensitive than culture. The sensitivity of Gram stain ranges from 40% to 90%, depending on whether the patient received antimicrobial therapy before lumbar puncture.

Latex agglutination tests for the detection of *H. influenzae* type b, *S. pneumoniae*, GBS, and *N. meningitidis* are available; however, these kits do not detect group B meningococci and coagulase-negative staphylococci. False-positive results can occur because of cross-reactivity with other bacterial species. False-negative results have been observed in specimens from pregnant women and infants. Routine use of antigen detection methods for diagnosis of bacterial meningitis is of limited value because the performance of the antigen test is similar to that of Gram stain, and a positive antigen test result usually does not alter the course of therapy. However, bacterial antigen testing may be beneficial in cases in which cultures are negative and clinical suspicion of bacterial meningitis is high or in cases of partially treated meningitis with sterile cultures. Multiplex PCR assays for the simultaneous detection of *N. meningitidis*, *H. influenzae*, and *S. pneumoniae* are available. These multiplex PCR assays are used in conjunction with traditional detection methods.

Viral infections

In viral infections, the number of lymphocytes in CSF is increased. The diagnosis of viral meningitis is based on detection of viral genome by PCR assay or detection of antigens by fluorescent antibody or enzyme immunoassay (EIA). The sensitivity of viral culture is only 14% to 24% compared with 88% to 94% with PCR, making PCR the method of choice for detection of viral causes of meningitis. The sensitivity and specificity of PCR assays can vary in relation to the virus being tested. Cell culture is recommended for EVs. However, due to cost, expertise required, and lengthy turn-around time, cell cultures are rarely performed in clinical microbiology laboratories. Recently, the GeneXpert EV assay (Cepheid), a real-time multiplex PCR assay for the detection of EV RNA in CSF, has become available. A positive result with the GeneXpert EV assay does not rule out other causes of meningitis. The results from this assay should be interpreted in conjunction with available clinical and laboratory information.

Real-time PCR assays are available for detection of PeV. For HSV, diagnosis includes detection of HSV DNA in CSF by PCR assay, growth of HSV on culture, antigen detection in brain biopsy samples, and demonstration of antibody in CSF and serum. Antigen and antibody assays have low sensitivity and are positive in the later stage of the disease. Although the PCR assay is highly sensitive, a false-negative result may be obtained in CSF samples obtained within the first 48 hours of illness. Repeated testing is helpful in such cases.

With infections caused by WNV, detection of IgM antibodies confirmed by the WNV plaque reduction neutralization antibody test is used for diagnosis, along with PCR and culture. The laboratory diagnosis of Japanese encephalitis virus is based on detection of viral RNA by PCR and virus-specific antibodies. Serum and CSF IgM antibody capture enzyme-linked immunosorbent assay (MAC-ELISA) has been widely accepted for the diagnosis of Japanese B encephalitis. However, the low specificity and cross-reactivity of the MAC ELISA has resulted in challenges in detection in areas in which Japanese encephalitis virus and other flaviviruses circulate simultaneously.

More recently, MassTag PCR, a multiplex PCR assay system with a library of primer sequences from a variety of pathogens that cause meningitis or encephalitis, has been developed. The tag from the amplified product is cleaved by ultraviolet light and is then analyzed by mass spectrophotometry. Chips that contain conserved sequences of viral genomes (DNA microarray) represent another new technology that is being developed for viral identification. In addition to the MassTag PCR and DNA microarrays, high-throughput DNA pyrosequencing using random primers has also been used to identify unknown viral pathogens.

Mycobacterial infections

Tuberculous meningitis is characterized by elevated lymphocyte count, elevated protein, and reduced glucose levels. The gold standard for diagnosis is identification of mycobacteria in the CSF by acid-fast staining and culture. Broth medium (e.g., Middlebrook 7H9, Dubos) and solid agar medium (e.g., Middlebrook 7H11, Löwenstein-Jensen) are used routinely to culture mycobacteria but require a long

incubation period (cultures held 6 weeks to 8 weeks). Use of the MGIT Mycobacterial Growth Indicator Tube (Becton Dickinson, Sparks, MD) or other automated systems, such as the MB/BacT (Organon Teknika, Durham, NC), ESP (Trek Diagnostic Systems, Westlake, OH), BACTEC9000 TB series, or BACTEC460 can increase recovery and reduce the time to detection of mycobacteria in CSF.

Direct staining is positive in 10% to 22% of cases, and cultures are positive in 38% to 88% of cases. The number of mycobacteria in CSF is usually small; therefore concentration of the CSF sample is useful in increasing recovery of the bacteria. Other techniques, such as the detection of mycobacterial antigen, antimycobacterial antibodies, or mycobacterial DNA by PCR assay, may be helpful. Commercial PCR assays are available for detection of *M. tuberculosis* in sputum and bronchial aspirates. These PCR assays have also been used for detection of mycobacteria in CSF; however, false-positive results do occur, and a negative test result does not exclude tuberculous meningitis. The PCR assays should be used in conjunction with culture.

Spirochetal infections

Lyme meningitis is also characterized by elevated lymphocyte, elevated protein, and reduced glucose levels. In symptomatic patients, diagnosis is made by using EIA or immunofluorescent assay followed by western immunoblot.

Fungal infections

Direct microscopic examination of Gram-stained or India ink-stained CSF samples, calcofluor-stained tissue samples, and culture are used to diagnose fungal causes of CNS infection. Concentration methods can increase the sensitivity of fungal staining and culture. Several media are available for fungal cultures of CSF or CNS tissue. All fungal cultures should be incubated for 4 to 6 weeks. Antigen detection by a latex agglutination test is useful in the case of infections caused by *C. neoformans* and *H. capsulatum*. The latex agglutination tests for *C. neoformans* show false-positive results with rheumatoid factor and in patients with infections caused by *Trichosporon* spp., *Stomatococcus mucilaginosus*, or *Capnocytophaga canimorsus*. False-negative results may be observed because of infection with a poorly encapsulated strain or low fungal burden. CSF and serum galactomanan levels are useful for the diagnosis of *Aspergillus* infections. Antibody detection using the complement fixation assay and immunodiffusion is useful for the diagnosis of *C. immitis*. PCR tests for routine detection of fungal DNA are not standardized.

Parasitic infections

The identification of parasitic causes of CNS infections is usually based on microscopic examination of Giemsa- or calcofluor-stained aspirate or biopsy samples (Fig. 35.13). Culture using agar with bacterial overlay is recommended for free-living amebae, such as *Naegleria* and *Acanthamoeba*, but not for *B. mandrillaris*. Serology and PCR testing are available for detection of *Toxoplasma*. Antibody detection by immunoblot is used as an adjunct serologic test for confirmation of radiologic diagnosis in patients with neurocysticercosis.

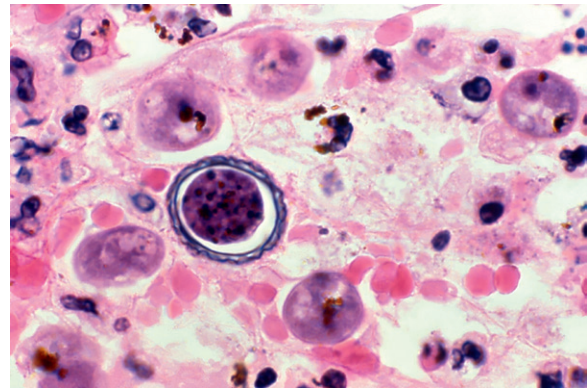


Fig. 35.13 This photomicrograph shows a magnified view of brain tissue, within which is a centrally located *Acanthamoeba* sp. cyst (hematoxylin and eosin stain, $\times 1000$). (Courtesy Dr. George Healy, Centers for Disease Control and Prevention, Atlanta, GA.)

Diagnosis of human trypanosomiasis is mainly via examination of unstained and Giemsa-stained blood smears, lymph node aspirates, bone marrow, or CSF for trypomastigotes. Other methods, such as the card agglutination trypanosomiasis test, various concentration techniques, and PCR, have been developed for the detection of trypanosomes but are mainly used as research tools. Concentration techniques using quantitative buffy coat analysis tubes increase detection sensitivity of the parasite. Hematocrit centrifugation and anion exchange column chromatography also increase the sensitivity of parasite detection. Examination of CSF processed by a double-centrifugation technique often reveals trypanosomes in patients in the late stage of the disease.

Case check 35.3

CSF samples should be transported to the laboratory without delay and processed as soon as possible to prevent loss of viability of the causative agent. In addition to routine laboratory testing, newer techniques for identification of viruses and other pathogens are becoming increasingly available. Rapid, accurate diagnosis of the causative agent is paramount in providing targeted therapy.

POINTS TO REMEMBER

- Host-related risk factors and pathogen-specific virulence factors are important in the pathogenesis of CNS infections.
- CSF characteristics, such as chemical and cellular features, may provide presumptive diagnostic clues about the cause of CNS infection.
- The age and immune status of the host will help determine likely causative pathogens.
- Virulence factors of disease-producing organisms enable them to evade host defense mechanisms and cause clinical infection.
- The prevalence of different viral pathogens can vary, depending on the season.
- The occurrence of opportunistic disease in patients with HIV infection is a function of underlying host immunodeficiency as well as pathogen virulence.
- Immunocompromised patients, including those who have undergone bone marrow or organ transplantation, are at increased risk for certain types of meningitis.

LEARNING ASSESSMENT QUESTIONS

- How is cerebrospinal fluid produced and distributed in the central nervous system?
- How would you characterize normal cerebrospinal fluid?
- Which common bacterial pathogens cause meningitis? Which host-related and virulence-related factors are associated with each pathogen?
- How would you compare the physical, chemical, and cellular findings in cerebrospinal fluid analysis in bacterial, fungal, tuberculous, and viral meningitis?
- Which fungal species are typically associated with intracerebral abscesses?
- Which fungal species are associated with meningitis?
- Why should you examine CSF specimens as soon as they are received?
- How would you initially evaluate the CSF sample?
- Which types of culture media should be used to recover bacterial pathogens?
- Which rapid and other ancillary methods are useful for evaluating CNS infections?
- An 18-year-old recruit is examined at the military health clinic. He is disoriented with a fever, intense headache, stiff neck, vomiting, and sensitivity to light. His roommates at the barracks say that he has been sick for about 2 days and that his condition worsened over the past 3 hours. The patient's serum electrolytes are normal, but the white blood cell count is 12,000 cells/ μ L. Which test should the health care provider order next?
 - Urinalysis and urine culture
 - Stool Gram stain and culture
 - Cerebrospinal fluid Gram stain and culture
 - Sputum Gram stain and culture
- Cerebrospinal fluid specimens should be analyzed for infectious agents as quickly as possible. How should a specimen collected in the hospital be transported to the laboratory?
 - Wet ice
 - Dry ice
 - At room temperature
 - Transport medium
- What are the most common bacteria isolated from nontraumatic brain abscesses?
 - Streptococci
 - Staphylococcus aureus*
 - Bacteroides* spp.
 - Escherichia coli*
- The direct Gram-stained smear of cerebrospinal fluid taken from a 4-week-old infant shows gram-positive cocci. Which of the following organisms would be highly suspected?
 - Escherichia coli*
 - Streptococcus pneumoniae*
 - Listeria monocytogenes*
 - Streptococcus agalactiae*
- A hunter is bitten by a raccoon. He washes the wound with soap and water and then puts a dressing on it. A few days

later, he notices that the wound is swollen and feels painful. He begins to experience seizures and hallucinations. Which condition corresponds with the hunter's symptoms and history?

- West Nile virus encephalitis
- Rabies
- St. Louis encephalitis (SLE)
- Colorado tick fever

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Bacteremia and sepsis

Paula C. Mister and Donald C. Lehman

CHAPTER OUTLINE

General concepts related to bacteremic infections, 905

Definitions, 905

Classification of bacteremia, 906

Epidemiology, 906

Incidence and mortality, 906

Risk factors, 907

Causes, 908

Pathogenesis, 909

Clinical aspects of bacteremia, 909

Syndromes associated with bacteremia, 909

Signs and symptoms, 910

Laboratory diagnosis, 911

Specimen collection, 911

Blood culture methods, 913

Blood culture systems, 914

Recovery of other types of organisms from blood, 916

Contamination in blood cultures, 917

Rapid identification of microorganisms growing in blood cultures, 917

Biomarkers, 918

Treatment, 919

Antimicrobial therapy, 919

Antisepsis therapy, 920

Prevention, 920

Bibliography, 921

6. Evaluate the significance of organisms commonly recovered from blood culture samples.
7. Describe the proper procedure for blood culture collection, timing of blood culture collection, and importance of adequate blood volumes for blood culture collection.
8. Describe the methods for the detection of bacteremia, including the following:
 - Media
 - Manual and automated blood culture systems
 - Blood culture additives, including substances to remove antimicrobials
 - Advantages and disadvantages of each system and additive described
9. Justify the methods used to diagnose viremia, fungemia, and fastidious and unusual bacteria in the bloodstream.
10. Describe the treatment modalities for bacteremia, sepsis, and septic shock.

KEY TERMS

Antimicrobial removal device (ARD)

Bacteremia

Community-acquired bacteremia

Continuous bacteremia

Disseminated intravascular coagulation (DIC)

Fungemia

Healthcare-associated bacteremia

Intermittent bacteremia

Occult (unsuspected) bacteremia

Polymicrobial bacteremia

Primary bacteremia

Pseudobacteremia

Secondary bacteremia

Sepsis

Septicemia

Septic shock

Sodium polyanethol sulfonate (SPS)

Systemic inflammatory response syndrome (SIRS)

Transient bacteremia

Viremia

OBJECTIVES

After reading and studying this chapter, you should be able to:

1. Differentiate bacteremia from septicemia.
2. Classify each type of bacteremia including when each condition occurs.
3. Discuss the epidemiology and pathogenesis of bacteremia.
4. Associate specific organisms with each type of bacteremia.
5. Explain the pathophysiology of sepsis and septic shock.

Case in point

A 4-year-old male patient with acute lymphocytic leukemia was undergoing induction chemotherapy administered via an

indwelling Hickman catheter and was hospitalized because of fever and granulocytopenia. His admission white blood cell (WBC) and platelet counts were $6 \times 10^9/\text{L}$ (reference range 4.5 to $11 \times 10^9/\text{L}$) and $55 \times 10^9/\text{L}$ (reference range 150 to $400 \times 10^9/\text{L}$), respectively, and his oral temperature was 103°F (39.4°C), reference range 95.9°F (35.5°C) to 99.5°F (37.5°C). Two sets of blood cultures were drawn before antimicrobial agents were administered. He was immediately given ceftazidime and vancomycin. All four blood culture bottles yielded gram-positive cocci in clusters after 24 hours of incubation. The isolate was identified as a coagulase-negative *Staphylococcus* sp. Removal and culture of the Hickman catheter and culture tip yielded the same organism with the same susceptibility pattern as that isolated from blood. Therapy was switched to more appropriate agents.

Issues to consider

After reading the patient's case history, consider:

- Conditions that predispose patients to bacteremic episodes
- Consequences of bacteremia caused by gram-negative and gram-positive organisms
- Source site where the organisms originate
- Appropriate blood culture method for isolation and recovery of the infecting agent

Bacteremia is the presence of viable bacteria in the bloodstream. Although bacteremia may be a transient, self-limiting phenomenon without clinical consequences, it frequently reflects the presence of serious infection. Life-threatening infections caused by bacteremia are a concern in patients who are immunocompromised because of drug or chemotherapeutic intervention or as the result of preexisting disease and subsequent immunosuppression. Bacteremia is often associated with hospitalization, insertion of foreign bodies, such as catheters into blood vessels, and other types of procedures.

This chapter begins with general concepts pertinent to bacteremic infections including definitions of conditions relating to bacteremia and how each condition is manifested, and then discusses the following:

- The epidemiology of risk factors for and pathogenesis of bacteremia
- Infections associated with bacteremia
- Diagnostic laboratory procedures
- Treatment modalities

General concepts related to bacteremic infections

Definitions

The presence of viable bacteria in the blood, as determined by their growth in a blood culture, is known as **bacteremia**. It is a laboratory finding that may or may not indicate infection. Blood cultures may become positive because of contamination during phlebotomy, leading to false-positive results, a phenomenon termed **pseudobacteremia**. Skin commensals, such as coagulase-negative staphylococci (CoNS), are common contaminants. Pseudobacteremia is not an indication of infection and does not require therapy. However, growth of

CoNS or other skin microbiota from a blood culture does not always represent pseudobacteremia; it may indicate true bacteremia, depending on the clinical situation.

Even when growth of an organism from a blood culture reflects a true-positive result, bacteremia may not be associated with any physical signs or symptoms of severe infection, a condition known as **occult (unsuspected) bacteremia**. Occult bacteremia frequently occurs in children younger than 2 years of age and is most often caused by *Streptococcus pneumoniae*. Because of the lack of clinical evidence for serious infection in such patients, the diagnosis of bacteremia may be overlooked, with potentially catastrophic consequences if treatment is delayed.

Usually, however, bacteremia that reflects true infection results in systemic physiologic responses that indicate the presence of a serious infection. In the past, the term **septicemia** was used to indicate bacteremia plus a clinical presentation of physical signs and symptoms of bacterial invasion and toxin production. The term *septicemia* is still used clinically and in the collection of epidemiologic data on causes of death. Because of its imprecision in defining a disease state, however, it is not suitable for categorizing all patients who have bacteremia-related infections or for designing clinical trials.

To facilitate the study of the pathogenesis and treatment of the consequences of severe infections, including those associated with bacteremia, an international consensus conference of the American College of Chest Physicians and the Society of Critical Care Medicine devised standardized definitions of the response to infection, shown in [Table 36.1](#). **Systemic inflammatory response syndrome (SIRS)** comprises a spectrum of increasingly severe conditions ranging from noninfectious inflammatory response to **sepsis** (infection with a systemic inflammatory response) to severe sepsis (sepsis accompanied by organ dysfunction, hypotension, or tissue hypoperfusion) to **septic shock** (sepsis accompanied by refractory hypotension). As might be expected, the risk of death progressively increases as patients move along this continuum. Although bacteremia may result in sepsis, severe

Table 36.1 Definition of terms related to severe infection

Term	Definition
Systemic inflammatory response syndrome	Systemic response to a variety of clinical insults. Criteria include at least two of the following: • Temperature $>38^\circ\text{C}$ or $<36^\circ\text{C}$ • Heart rate >90 beats/min • Respiratory rate >20 breaths/min, or partial pressure of carbon dioxide (PaCO_2) <32 mm Hg • White blood cell count $>12 \times 10^9/\text{L}$ or $<4 \times 10^9/\text{L}$, or $>10\%$ band forms
Sepsis	Infection with systemic inflammatory response
Hypotension	Systolic blood pressure <90 mm Hg, mean arterial pressure <70 mm Hg, or reduction >40 mm Hg from baseline
Severe sepsis	Sepsis associated with organ dysfunction, hypoperfusion, or hypotension
Septic shock	Sepsis with hypotension despite adequate fluid resuscitation and requiring pressor therapy, along with perfusion abnormalities

sepsis, or septic shock, these responses are not automatically associated with bacteremia. For example, blood culture results may be negative in more than 70% of patients with sepsis despite clear clinical signs of infection.

Classification of bacteremia

Site of origin

Bacteremia may be classified by its site of origin. **Primary bacteremia** occurs when the bacteria are present in an endovascular source such as an infected cardiac valve or an infected intravenous (IV) catheter, whereas **secondary bacteremia** occurs when the bacteria come from an infected extravascular source, such as lungs in patients with pneumonia. A case in which the source of bacteremia remains undefined is termed *bacteremia of unknown origin*. Classification in this manner has important clinical consequences because it determines the appropriate therapy and prognosis. For example, a secondary bacteremia from an infected focus, such as an abscess, may require surgical therapy to remove the abscess or source of infection, in addition to antimicrobials to eliminate the infection. Bacteremia of unknown origin generally has a poorer prognosis than primary or secondary bacteremia.

Causative agent

Bacteremia may also be categorized by the general class of microorganism or specific pathogen that has invaded the bloodstream. Gram-positive bacteremia is caused by such organisms as *S. pneumoniae*, *Staphylococcus aureus*, or *Enterococcus faecium*, whereas gram-negative bacteremia is caused by such organisms as *Escherichia coli* or *Pseudomonas aeruginosa*. Anaerobic bacteremia is caused by such organisms as *Bacteroides fragilis*, whereas **polymicrobial bacteremia** is caused by a mixture of organisms. General classification of bacteremia in this fashion can provide initial clues to the underlying source of a bacteremia and guide therapy, even before organisms have been identified. For example, CoNS bacteremia in a hospitalized patient is frequently caused by infection of an indwelling vascular device, whereas polymicrobial bacteremia with a mixture of enterococci and gram-negative organisms is frequently caused by invasion of the bloodstream by gastrointestinal (GI) microbiota from bowel perforation.

Place of acquisition

Bacteremia can also be categorized by its place of acquisition. **Community-acquired bacteremia**, as the term suggests, occurs in individuals living in the general community, whereas **healthcare-associated bacteremia** occurs in patients who are hospitalized or living in a nursing home or other health care facility. To avoid misclassification of bacteremia that began at the time of hospital admission as healthcare-associated, when it is, in fact, community acquired, a healthcare-associated infection is conventionally defined as any bloodstream infection occurring more than 72 hours after hospital admission.

Certain bacteremias are more often community acquired. For example, more than 90% of cases of *S. pneumoniae* bacteremia are acquired in the community. Others, such as those caused by *P. aeruginosa* or *Enterococcus* spp., are more likely to be healthcare-associated. The place of acquisition may thus be extremely significant in guiding initial therapy. For example,

healthcare-associated bacteremia is more likely to be caused by drug-resistant organisms that express β -lactamases or other resistance factors that inactivate first-line antimicrobial agents, although this distinction is currently blurred in the case of hospital-acquired and community-acquired methicillin-resistant *Staphylococcus aureus* (MRSA).

Duration

Bacteremia may also be classified by the duration of a bacteremic episode. Bacteremic episodes may be *transient*, *intermittent*, or *continuous*. The frequency, time, and number of blood cultures to be collected may depend on the type of bacteremic episode that the patient is experiencing. **Transient bacteremia** usually occurs after a procedural manipulation of a specific body site colonized by indigenous microbiota, causing the organisms to enter the vascular system. Such sites include the mouth and the GI and urogenital tracts. Transient bacteremia may appear for a brief period following a dental, colonoscopic, or cystoscopic procedure. The organisms involved are normally rapidly cleared by the host immune defense, so their presence is rarely symptomatic.

Intermittent bacteremia can occur because of the presence of abscesses somewhere in the body or as a clinical manifestation of certain types of infections, such as meningococcemia, gonococcemia, or pneumonia. In intermittent bacteremia, organisms are periodically released from the primary site of infection into blood. **Continuous bacteremia** occurs when the organisms are coming from an intravascular source and are consistently present in the bloodstream. Infective endocarditis is the most common clinical manifestation associated with continuous bacteremia, although other endovascular sources, such as infected intravascular catheters or septic thrombi, can also result in continuous bacteremia. Microbial biofilms (see [Chapter 31](#)) on foreign body implants and in tissue can contribute to continuous bacteremia by the periodic release of planktonic microorganisms.

Epidemiology

Incidence and mortality

Brill reported the first case of bacteremia (caused by *Bacillus pyocyaneus*, now *P. aeruginosa*) in 1899. Ten years later, fewer than 40 cases had been reported worldwide, with fewer than 30 additional cases in the following 15 years. Bacteremia currently occurs in more than 250,000 hospitalized patients in the United States, but not all of these cases result in septicemia. Between 1950 and 2003, however, the mortality rate caused by septicemia increased almost 40-fold. Septicemia (as defined by the *International Classification of Diseases* [ICD]-10) was the 11th leading cause of death overall in the United States in 2019 (1.42 % of total deaths) based on data from the Centers for Disease Control and Prevention (CDC). In 2019, CDC reported bacterial sepsis as the seventh leading cause of death among newborns, with 16.1 deaths/100,000 live births.

Documented bacteremia is a fundamental determinant of septicemia, which often leads to shock and death. The incidence of septic shock in patients with bacteremia ranges from 10% to 30%, depending on the pathogen, source of bacteremia, presence of immunosuppression, and presence or absence of comorbid conditions, but once present, septic

shock is associated with a mortality rate of 30% to greater than 50%. According to the CDC, mortality rates in patients with bacteremia and fungemia currently remain as high as 12% and as high as 25% for candidemia specifically. Patients with severe sepsis and septic shock who have three or more failing organs have a mortality rate of 70%. Factors associated with an unfavorable outcome in bacteremia include the following: age older than 70 years; polymicrobial bacteremia; presence of malignancy, acquired immunodeficiency syndrome (AIDS), or renal failure; origin of the bacteremia in the respiratory tract or bowel; unknown origin of bacteremia; and delayed or inappropriate antimicrobial therapy.

Risk factors

The increased incidence of bacteremia in the United States during the past 30 years is likely a result of the following factors, some of which have changed dramatically over this period:

- Decreased immunocompetency of selected patient populations
- Increased use of invasive procedures
- Age of the patient
- Antimicrobial resistance
- Diagnostic criteria and coding practices
- Injection drug use
- Nasal colonization (in the case of *S. aureus* bacteremia)

Decreased immunocompetency of selected patient populations

Bacteremias are more frequent among persons with *neoplasia* (abnormal growth of new cells that may be benign or malignant), especially those with hematologic malignancies, those receiving immunosuppressive chemotherapy, and those undergoing bone marrow transplantations. This is caused, in part, by a decrease in the levels of circulating neutrophils in such patients as well as disruption in the GI mucosa attributable to chemotherapy, allowing invasion of normal microbiota into the bloodstream. Persons with other chronic underlying diseases (e.g., diabetes, cirrhosis) and those receiving immunosuppressive therapy (e.g., those receiving glucocorticoids for rheumatoid arthritis, stem cell or solid organ transplant recipients) are also at increased risk for bacteremia. Infection with human immunodeficiency virus (HIV) predisposes patients to increased risk of bacteremias because of the immunosuppression caused by the virus.

Increased use of invasive procedures

The increased use of indwelling devices, respirators, and invasive diagnostic procedures is a common cause of bacteremia and actually the leading cause of healthcare-associated bacteremia. The widespread use of semi-permanent vascular catheters (to administer chemotherapy to patients with cancer or to provide vascular access for hemodialysis in patients with end-stage renal disease) increases the risk of bacteremia by breaking the normal integrity of the skin and permitting colonization of a foreign body in direct contact with the bloodstream. According to the National Healthcare

Safety Network, more than 30,000 bacteremias (central line-associated bloodstream infections [CLABSI]) occur annually in the United States because of infected central line venous catheters, with a mortality rate of 12% to 25%. Indwelling urethral catheters, suprapubic catheters, and IV pyelography also predispose patients to catheter infections by penetrating otherwise sterile areas, encouraging colonization with bacteria from surrounding tissue. Surgery involving the urinary, GI, and biliary tracts may also result in bacteremia because of the disruption of mucosal barriers that normally function to block the spread of resident microbiota.

Age of the patient

Bacteremias are more prevalent in people at the extremes of age, with infants, young children, and adults older than 55 years of age being most susceptible. Both infants and older adults have decreased immune system function compared with people 5 to 55 years of age. In very young patients a competent immune response has not yet fully developed, and in older adults there is a general decrease in immunocompetency with age. The presence of comorbid conditions, such as diabetes, hypertension, chronic obstructive pulmonary disease, and congestive heart failure, in older adults and neoplastic disorders, HIV infection, and low and very low birth weights in neonates and infants significantly increases the incidence of bacteremia in these groups.

Case check 36.1

The patient in the Case in Point had several risk factors for developing bacteremia: he was immunocompromised (leukemic), was hospitalized, and had an indwelling catheter. Other risk factors for bacteremia include very young age, old age, and trauma. The increased number of persons with these risk factors partly explains the recent increase in bacteremias noted.

Antimicrobial resistance

The indiscriminate administration of broad-spectrum antimicrobials reduces susceptible normal microbiota and favors colonization and invasion by resistant bacteria. Data from the SENTRY Antimicrobial Surveillance Program (2012–2017) revealed that, on average, 37% of *S. aureus* isolates from bloodstream infections in the United States were resistant to methicillin (oxacillin), 26.4% of *Enterococcus* blood isolates were resistant to vancomycin, 15.8% of Enterobacterales blood isolates were multidrug resistant, 21.5% of *Klebsiella* spp. blood isolates were extended-spectrum β -lactamase (ESBL) producers, and 15.4% of *P. aeruginosa* blood isolates were multidrug resistant. These percentages vary widely from region to region and are higher in patients with healthcare-associated infections based on the WHO Global Antimicrobial Resistance and Use Surveillance System [GLASS] report, 2021.

Although the rates of *S. aureus* bloodstream infections occurring in hospitalized patients decreased by almost 50% from 1997 to 2007 and 54% between 2005 and 2011, the percentage of *S. aureus* isolates that were MRSA dramatically increased; therefore numbers of MRSA blood infections remained fairly constant. There were approximately 120,000 MRSA bloodstream infections documented in the United

States in 2017, resulting in 20,000 deaths. According to the CDC, the rate has decreased slowly, but community-acquired methicillin-sensitive *S. aureus* (MSSA) bacteremia has increased to 3.9% annually. An increasing population of antimicrobial-resistant organisms results in bacteremias that are harder to treat, leading to increased morbidity and mortality.

Diagnostic criteria and coding practices

The factors described have been shown to lead to an actual increase in the incidence of bacteremia. There may be some artificial increase in the incidence of bacteremia because of variations in the diagnostic criteria for bacteremia and how bacteremias are coded in the patient record. It is difficult to compare rates from one study to another because of these changes over time (e.g., pre-consensus conference to post-consensus conference changes) and changes from one health care institution to another and one health care provider to another. In addition, there are multiple ICD codes that may apply to bacteremia and/or fungemia; for example, bacteremia, septicemia, disseminated fungal infection, disseminated candidal infection, and disseminated fungal endocarditis are all assigned separate ICD-10 codes. Several of these codes overlap and could potentially apply to the same infection. Investigators in different studies may consider some or all of these when evaluating rates of bacteremia and fungemia. Finally, each revision of the ICD is slightly different. Since 2004, hospitals have been using the 10th revision, second edition, of the ICD.

Causes

Over the past 25 years, the pattern of organisms responsible for bacteremia has shifted. In the 1960s and 1970s, gram-negative organisms, such as *E. coli* and *P. aeruginosa*, predominated in prospective studies of bacteremia. In the 1980s and 1990s, the pattern shifted such that most bacteremias in the United States were caused by gram-positive organisms, such as *S. aureus*, CoNS, and *Enterococcus* spp., although gram-negative organisms still represented a large proportion of cases. A SENTRY 20-year trend report (2019) states that bacteremias caused by gram-negative organisms are again increasing, with high rates of gram-negative infections occurring in infants (up to 42%). Fungal invasion of the bloodstream (**fungemia**) caused by such organisms as *Candida albicans* and *Candida auris* has become increasingly important.

In recent data from the CDC (2021), the most common causative bloodstream pathogens, in descending order, from catheter-related bloodstream infections (CRBSIs) are CoNS, enterococci, *S. aureus* (both MSSA and MRSA), aerobic gram-negative bacilli, and *C. albicans*. Table 36.2 shows the frequencies of bacteremia caused by different organisms in a 2004 hospital-based prospective survey of healthcare-associated bacteremias (most commonly catheter related). Recent shifts in the microbiology are likely a result of risk factors discussed earlier: widespread use of indwelling vascular catheters (readily colonized by CoNS); immunosuppressive cancer chemotherapy, which renders patients susceptible to infection by CoNS and other commensals that normally do not cause disease in immunocompetent individuals; and increasing antibiotic drug resistance in gram-negative rods.

Table 36.2 Most common organisms associated with hospital-acquired bacteremias

Organism	Percentage of bacteremias (N = 20,978)
Coagulase-negative staphylococci	31.3
<i>Staphylococcus aureus</i>	20.2
<i>Enterococcus</i> spp.	9.4
<i>Candida albicans</i>	9.0
<i>Escherichia coli</i>	5.6
<i>Klebsiella</i> spp.	4.8
<i>Pseudomonas aeruginosa</i>	4.3
<i>Enterobacter</i> spp.	3.9
<i>Serratia</i> spp.	1.7
<i>Acinetobacter baumannii</i>	1.3

Data from Wisplinghoff H., et al. (2004). Hospital-acquired bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. *Clinical Infectious Diseases: an Official Publication of the Infectious Diseases Society of America*, 39, 309.

In addition to the shift in organisms responsible for bacteremia, the susceptibility patterns of pathogens causing bacteremia have also changed. Bacteremia caused by MRSA, a rare entity in the 1970s, now accounts for more than 40% of all hospitalizations for *S. aureus* bacteremia, and the incidence of bacteremia caused by vancomycin-resistant enterococci (VRE) has also increased. In addition, gram-negative organisms expressing ESBLs and carbapenem-resistant Enterobacterales (CRE) have become more prevalent, complicating the therapy of patients with bacteremia resulting from these organisms. These shifts are thought to be caused by the increasing use (and misuse) of broad-spectrum antimicrobials, resulting in selection of multidrug-resistant pathogens.

The microbiology of bacteremia is also marked by an increasing incidence of polymicrobial bacteremia. In the 1930s, almost every case of bacteremia involved a single organism. By the early 1990s, 10% of bacteremias involved more than one organism. Polymicrobial bacteremia is generally associated with a higher mortality than monomicrobial bacteremia. The predisposing factors in polymicrobial bacteremia include IV drug use, burns, and GI tract sources. Especially at risk are immunocompromised patients, particularly those with alcoholism, granulocytopenia, extensive burns, diabetes mellitus, and chronic renal failure, and patients with vascular insufficiency attributable to ischemia. *B. fragilis* often has been associated with polymicrobial infections. Given the resistance of *B. fragilis* to many antimicrobials, its involvement in bacteremia carries an increased risk for death.

Finally, some organisms have become less prevalent causes of bacteremia because of immunization practices that have decreased the risk of infection. For example, the incidence of infection with *Haemophilus influenzae* type b (Hib), formerly a major cause of bacteremia and sepsis in children, decreased by more than 95% after introduction of the conjugate Hib vaccine in the 1980s. Similarly, implementation of the *S. pneumoniae* vaccine in the 1970s resulted in an 84% reduction of bacteremia caused by *S. pneumoniae* as well. Interestingly, according to one study, there has been a 67%

reduction in bacteremia in general in 3- to 36-month-old children. *S. pneumoniae* now accounts for <2% of bacteremias (SENTRY 20-year trends, 2019).

Pathogenesis

The pathogenesis of bacteremia depends, in part, on the infecting pathogen, portal of initial entry, and immune status of the patient. In general, however, bacteremia occurs because of disruption of normal skin or mucosal barriers, leading to bacterial invasion of the bloodstream. Such disruption may occur because of trauma, burns, or ischemia giving rise to breaks in the skin that allow access to the microvasculature; an antecedent viral infection that disrupts the epithelial lining (e.g., influenza virus infection involving the upper respiratory tract) and allows resident biota to invade the bloodstream via capillaries; or iatrogenic disruptions, such as surgery, instrumentation, or placement of an indwelling device. Alternatively, a focal bacterial infection (e.g., bacterial pneumonia) may lead to bacteremia via local inflammation, edema, and tissue destruction that disrupts nearby vascular structures and allows bloodstream invasion.

Once bacteremia occurs, the patient's immune system attempts to control infection via antibodies that opsonize organisms and activate complement-mediated killing and by phagocytosis. In addition, filtering mechanisms in the lymphatics and large vascular beds in the liver and spleen may sequester organisms and facilitate their destruction by phagocytic cells. If, however, these defenses are unsuccessful, two major complications may ensue—metastatic infection and septic shock. Invasion of the bloodstream may result in spread of organisms throughout the body, causing seeding of multiple sites and leading to widely disseminated infection. For example, bacteremia caused by *S. pneumoniae* can lead to infection of the meninges, resulting in pneumococcal meningitis, a catastrophic infection with a mortality rate as high as 25%, even with optimal treatment. Other infections associated with a period of bacteremia as part of the disease process include salmonellosis, infective endocarditis, and acute hematogenous osteomyelitis. *S. aureus* is particularly likely to cause metastatic infection or abscess formation as a consequence of bacteremia; *S. aureus* bacteremia may lead to endocarditis, osteomyelitis, septic arthritis, hepatic abscess, or pyomyositis. Sepsis and septic shock are also potential consequences of bacteremia.

Although gram-negative bacteremias were once thought to be more likely to cause septic shock than bacteremias caused by gram-positive organisms, the risk of sepsis, severe sepsis, septic shock, and death is now known to be similar between these two classes of bacteremia. In both cases, a bacterial membrane component (lipopolysaccharide, also known as *endotoxin*, in gram-negative organisms, lipoteichoic acid and peptidoglycan in gram-positive organisms) interacts with macrophages and causes the release of tumor necrosis factor, interleukin (IL)-1, IL-6, and other proinflammatory cytokines, increasing endothelial activation, vascular permeability, blood flow, and recruitment of neutrophils. These responses are directed at controlling infection and are normally counterregulated by anti-inflammatory mediators to prevent a destructive systemic inflammatory reaction. In sepsis and septic shock, however, an imbalance in regulation leads to an

unopposed proinflammatory state, leading to microvascular abnormalities and endothelial injury; in turn, these derangements lead to decreased tissue perfusion, complement activation, and **disseminated intravascular coagulation (DIC)**, which cause multiorgan dysfunction, eventually leading to septic shock and death.

Clinical aspects of bacteremia

Syndromes associated with bacteremia

Numerous sources can give rise to bacteremia. The most common sites associated with bacteremia and sepsis are infected intravascular catheters, urinary tract, lung, and abdomen.

Catheter-related bloodstream infections

Intravascular catheters have become indispensable for modern medical and surgical therapy. Most of these are temporary catheters placed in peripheral veins and are intended for short-term use in the administration of medications and fluids. Semi-permanent catheters (Hickman, Broviac, Quinton, or Tenckhoff catheters, so named after their developers) are placed in central veins and remain in place for weeks or months to administer chemotherapy and/or parenteral nutrition to patients with cancer or to perform hemodialysis in patients with end-stage renal disease. Still other catheters are placed in arteries or, via central veins, the pulmonary artery or right-sided chambers of the heart, where they may be left for days for hemodynamic monitoring.

As useful as these devices are, they are exquisitely vulnerable to colonization and biofilm formation by gram-positive organisms, such as CoNS, *S. aureus*, and *Enterococcus*, which are often found on the surface of the patient's skin, with subsequent spread to the bloodstream to cause bacteremia. Frequently the strains causing CRBSIs are resistant to multiple antimicrobials. Substantial progress has been made in the past 20 years, however, in reducing these infections. CDC data revealed more than 30,000 cases of documented CRBSI in the United States in 2018 in hospital patients, a 58% reduction since 2001. Most of these infections are caused by CoNS. Production of a polysaccharide biofilm by CoNS is one mechanism that has been associated with CRBSI. The biofilm may serve as a ligand during initial surface adhesion and colonization, or it may be produced after the organism has established a focal presence by adhering to the surface. The biofilm also protects the organism from host defenses by inhibiting phagocytosis, chemotaxis, and oxidative metabolism and by suppressing the lymphoproliferative response. It may also significantly increase the concentrations of antimicrobials required to inhibit the growth of CoNS attached to the catheter.

Although CoNS are of relatively low virulence, bacteremias caused by these organisms in patients with compromised immune systems (e.g., patients with cancer becoming neutropenic through chemotherapy) are associated with mortality rates of 13% to 18%. Bacteremias caused by *S. aureus* are associated with higher mortality rates, partly because of the virulence of this organism and the ease with which it adheres to native cardiac valves and causes endocarditis. Occasionally,

fluids administered via these catheters become contaminated via a break in infusion lines, causing bacteremia. Organisms associated with such infusion-associated bacteremias are typically gram-negative organisms, such as *P. aeruginosa* and *Enterobacter cloacae*. Bloodstream infections caused by nontuberculous mycobacteria associated with intravascular catheters are rapidly emerging among immunosuppressed hosts, most notably *Mycobacterium avium* complex in HIV-positive individuals.

Case check 36.2

The Case in Point is a classic example of a CRBSI with CoNS, weakly virulent organisms that can be pathogenic in a high-risk patient. Bacterial contamination via the catheter likely introduced the organism into the bloodstream. This was demonstrated by recovery of the same organism with the same susceptibility from culture of the catheter and the blood. Removal of the catheter was the clinically appropriate action.

Urinary tract infections

Infection of the upper urinary tract (acute pyelonephritis) leads to bacteremia in as many as 40% of affected patients. *E. coli* is the predominant cause of bacteremia in this setting. These infections are most common in older adult patients.

Pneumonias

Common organisms in cases of pneumonia that produce a concurrent bacteremia include *S. pneumoniae*, *S. aureus*, *P. aeruginosa*, *Klebsiella* spp., and *Enterobacter* spp. Of patients with pneumococcal pneumonia, 25% to 30% will have positive blood cultures; the case fatality rate is 5% to 7%, and likely much higher in older adult patients.

Intraabdominal infections

Primary peritonitis, which frequently occurs in patients with cirrhosis, is associated with bacteremia in 75% of cases involving aerobic bacteria. Common pathogens include *E. coli*, *K. pneumoniae*, and enterococci. Secondary peritonitis arising from perforation of the GI tract may give rise to intraabdominal abscesses, resulting in intermittent bacteremia caused by *E. coli*, anaerobes, and enterococci. In some cases, intraabdominal abscesses may be caused by seeding of the liver or spleen by a transient bacteremia arising from outside the abdomen, most often caused by *S. aureus*.

Skin infections

Cellulitis caused by *S. aureus*, *Streptococcus pyogenes*, or *Streptococcus agalactiae* can lead to bacteremia. Skin breakdown in bedridden patients (bed sores or decubitus ulcers) or peripheral vascular disease from diabetes is a common cause of infected skin ulcers, which can provide a portal of entry for bacterial invasion of the bloodstream, often resulting in polymicrobial bacteremia. Commonly reported offending organisms include *Proteus mirabilis*, *E. coli*, *S. aureus*, *B. fragilis*, *Pseudomonas* spp., *Clostridium* spp., and

Peptostreptococcus. Surgical treatment of abscesses and wounds can result in bacteremia. Patients with severe burns who become bacteremic most often grow gram-negative organisms, especially *P. aeruginosa* and *Klebsiella pneumoniae*. IV drug use, under unsterile conditions, can also result in bacteremia by introducing skin microbiota, environmental organisms, or mouth biota (from licking needles) into the bloodstream.

Infective endocarditis

Transient bacteremia (from dental procedures or a superficial skin infection) can seed cardiac valves with bacteria. These organisms multiply within a dense vegetation composed of bacteria and fibrin, protected from killing by neutrophils, and give rise to a continuous bacteremia that can then seed other organs. Continued growth can lead to valvular damage and congestive heart failure. Organisms associated with acute, sudden-onset endocarditis include virulent bacteria, such as *S. aureus*, enterococci, and *S. pneumoniae*, whereas the more slowly progressing subacute endocarditis is commonly caused by less virulent bacteria, such as viridans streptococci; nutritionally variant streptococci, including *Abiotrophia* and *Granulicatella*; and CoNS (particularly in prosthetic heart valves).

Musculoskeletal infections

Acute osteomyelitis is often associated with transient bacteremia caused by *S. aureus*; frequently the portal of entry is an otherwise unnoticed minor skin infection. The organisms seed end loop capillaries in bone, where blood flow is slow, and begin to multiply, causing destruction of bone and giving rise to intermittent bacteremia in about 50% of cases. Prosthetic joints, particularly those implanted in the hip, can be hematogenously seeded by such organisms as *S. aureus* and CoNS and then give rise to bacteremia. Occasionally, prosthetic joint infections are caused by intraoperative contamination, although this risk has decreased considerably because of the use of perioperative prophylaxis and ultra-clean operating rooms. Prosthetic joint infection with virulent organisms such as *S. aureus* or group A β -hemolytic streptococci can lead to florid sepsis and death. Bacteremia caused by *S. aureus* or *Neisseria gonorrhoeae* can result in seeding of joints and cause acute septic arthritis, an infectious disease emergency that requires prompt drainage of pus from the joint and aggressive antimicrobial therapy.

Central nervous system infections

Acute bacterial meningitis is generally the result of transient bacteremia caused by *S. pneumoniae* or *Neisseria meningitidis*. These organisms may colonize the nasopharynx in normal individuals and occasionally invade the bloodstream to cause disease. Occasionally, meningitis is caused by bacteremia resulting from sinusitis or otitis caused by *S. pneumoniae*.

Signs and symptoms

The classic signs and symptoms of bacteremia include abrupt onset of shaking chills, fever, or hypothermia. About 14% of patients with bacteremia will have hypotension, and about

40% of patients experience prostration and diaphoresis (profuse sweating). Tachypnea (abnormal rapid breathing) is an early sign of bacteremia, and adult respiratory distress syndrome occurs in 18% of patients with culture-positive septic shock. Other symptoms may include delirium, stupor, or agitation (evidence of decreased central nervous system perfusion), along with nausea and vomiting. As many as 38% of patients with bacteremia and sepsis will have acute renal failure, with oliguria or anuria. Ecthyma gangrenosum, a central necrotic area surrounded by an erythematous base, is typically associated with *Pseudomonas* bacteremia. The failure of the body to mount an elevated temperature is associated with increased mortality in newborns and older adults. Clinical conditions with altered laboratory values that may be indicative of bacteremia include the following:

- Thrombocytopenia
- Leukocytosis or leukopenia
- Lactic acidosis
- Hypoglycemia or hyperglycemia
- Abnormal liver function test results (especially hyperbilirubinemia)
- Coagulopathy, including DIC
- Elevations in C-reactive protein (CRP), haptoglobin, and fibrinogen levels

Laboratory diagnosis

Specimen collection

Because of the potential of a serious negative outcome of a septicemia or bacteremia, a blood culture is one of the most important cultures to obtain rapid accurate results. As such, a positive blood culture is a critical value (Box 36.1). Critical values are laboratory test results that are outside of the reference range and indicate a potentially fatal outcome. The health care provider must be notified immediately of all critical values to provide immediate treatment. In the case of a positive blood culture, the Gram stain result should be called to the health care provider immediately.

BOX 36.1 Critical values

The following must be communicated immediately to the health care provider:

- Positive Gram-stained smear result from bottle for bacterial or yeast blood culture
- Positive acid-fast smear result from bottle for mycobacterial blood culture

Some facilities consider the following critical values that must be communicated (but others do not):

- Positive direct coagulase from blood culture bottle result (indicates presence of *Staphylococcus aureus*)
- Positive Quellung capsule staining result (presumptive *S. pneumoniae*)
- Positive modified Kinyoun stained smear result (presumptive *Nocardia* sp.)
- Positive peptide nucleic acid fluorescence in situ hybridization result for yeasts, *Enterococcus* sp., or *S. aureus*
- Definitive identification of *Mycobacterium tuberculosis* from blood culture

It is important to remember that even though antiseptic technique is used in the collection of blood, somewhere between 1% and 3% of blood cultures become contaminated with such organisms as CoNS, *Corynebacterium* spp., *Bacillus* spp. (not *B. anthracis*), α -hemolytic streptococci, and *Cutibacterium acnes*, which are ordinarily skin colonizers, resulting in pseudobacteremia. However, in some patients, including those undergoing cancer chemotherapy, such organisms can represent true pathogens, making it essential to distinguish between blood culture results that reflect true bacteremia and those that represent pseudobacteremia.

Therefore it is most important to prepare the skin properly before venipuncture for blood culture. Palpation for the vein can be checked with a gloved finger. Cleansing the skin with 70% to 95% ethanol or isopropyl alcohol followed by 2% chlorhexidine (preferred) or 2% tincture of iodine, scrubbed in a concentric fashion around the venipuncture site is recommended. For decontamination to be effective, the antiseptic should be left on the skin for at least 30 seconds. After the venipuncture, the disinfecting agent should be removed with an alcohol pad. Because of systemic absorption and their effect on thyroid function, iodinated antiseptics should not be used on low-birth-weight infants. Similarly, in infants younger than 2 months of age, chlorhexidine should not be used; instead, alcohol swabs should be used.

When blood is collected for culture, it is critical that the blood not be allowed to clot. The formation of a clot will trap the bacteria and reduce the ability to detect them. Thus blood should be inoculated directly into blood culture bottles or, if necessary, into a tube containing anticoagulants. Tubes containing heparin, ethylenediaminetetraacetic acid (EDTA), and sodium citrate have been shown to inhibit the growth of many different organisms and should not be used. Tubes containing 0.025% to 0.050% **sodium polyanethol sulfonate (SPS)** are better tubes to use for collecting blood for culture, although SPS can inhibit a few organisms as well, notably *Peptostreptococcus anaerobius* and some strains of *Neisseria* spp. and *Streptobacillus moniliformis*. Intermediate collection tubes are therefore not recommended.

Blood for blood culture should be obtained by venipuncture and not from indwelling IV or intraarterial lines. There is more risk of isolating skin microbiota from indwelling lines rather than via venipuncture. If blood is collected from an indwelling line, a second sample collected via venipuncture should be cultured for comparison. For patients who have IV lines through which they are getting fluids and/or medications, blood must be drawn below the line because blood drawn above the line will be diluted with the fluid being transfused. The best practice, however, is not to draw blood from the extremity that has an IV line but to perform venipuncture on an extremity that does not have an indwelling line.

Determining the volume, frequency, and number of blood cultures

Density of bacteremia in adults versus neonates

Bacteremia may involve a large number of microorganisms in the blood; however, more commonly, only a relatively small number of bacteria per unit volume of blood (as low as one bacterium per milliliter) is seen in patients with clinically significant bacteremia. The detection methods commonly

used in most laboratories produce positive results in adult patients with bacteremia if organisms are present in the range of 10 to 15 bacteria per milliliter of blood; however, there are circumstances in which patients have fewer than this number.

Studies that have examined the relationship between the volume of blood collected and rate of positivity have demonstrated that as the volume of blood cultured is increased from 2 to 20 mL per culture, the yield of positive culture results increases from 30% to 50%. One study clearly demonstrated the advantage of culturing 10 mL of blood per blood culture bottle compared with 5 mL of blood per bottle, finding that 7.2% more cases of bacteremia were detected in the bottle inoculated with 10 mL of blood and that the organisms were detected sooner than in the bottles inoculated with only 5 mL of blood. Unfortunately, a number of studies have found that it is common practice to inoculate blood culture bottles with less than the recommended volumes of blood.

If inoculating more blood into the blood culture bottle increases the sensitivity and rate of detection, why not inoculate even more blood than recommended into each bottle? Taking excessive amounts of blood from a patient can induce anemia in the patient, and according to at least one study, inoculating more than 10 mL per bottle did not increase the rate of positivity and decreased it instead.

Blood naturally contains many inhibitors of bacterial growth, from complement and lysozyme to WBCs. When more blood is inoculated into the blood culture bottle, the amount of inhibitors in the bottle is also increased, resulting in fewer bacteremic episodes detected in culture. In addition, although blood should be collected before antimicrobial agents are given, it is likely that many patients from whom blood is drawn are already receiving antimicrobial treatment that also inhibits the growth of bacteria in blood culture bottles. Thus blood and the inhibitors present in blood need to be diluted by the blood culture medium. The optimal ratio of blood to culture medium is about 1:5 to 1:10. The dilution aids in negating the bactericidal effect of normal serum. In cases in which a 1:5 dilution cannot be achieved, 0.025% to 0.050% SPS can be added to the bottle; this serves to inhibit complement, coagulation, and phagocytosis within the bottle.

In newborns, sepsis tends to be more severe because of their immature immune defense mechanisms and generally higher numbers of microorganisms per milliliter of blood. It is not uncommon, however, to see low-level bacteremia in children as seen in adults. Because newborns and children have a smaller volume of total blood, it is not safe to take as much blood from a newborn or child as that which can be taken from an adult. As a rule, about 4% of an adult patient's total blood volume can be taken safely for culture, but there is controversy over appropriate volumes to collect from infants and young children. In 2007, the Clinical and Laboratory Standards Institute (CLSI) recommended that no more than 1% of total blood volume be drawn for culture in infants and young children. Age-volume protocols used in the past are not recommended; these have become highly inaccurate in the present-day population. A protocol published by the American Society for Microbiology (ASM) in 2005 used the relationship between weight and blood volume to determine how much blood can be drawn for culture in this age group, assuming that similarly to adults, up to 4% of blood volume can be safely obtained.

In this scheme, 2 mL of blood can be safely collected from a newborn weighing less than 1 kg (<2.2 lb), 4 mL total from a newborn weighing up to 2 kg (up to 4.4 lb), 6 mL total from a child weighing up to 12.7 kg (27 lb), and 10 mL total from a child weighing up to 36.3 kg (up to 80 lb). A child who weighs more than 36.3 kg (>80 lb) can have as much blood drawn as an adult (20 to 30 mL). Because less blood is typically drawn from newborns and children compared with adults, most commercial blood culture systems have pediatric bottles that contain less blood culture medium into which less blood is inoculated. The ratio of blood to broth is kept at 1:5 to 1:10; some pediatric bottles may be supplemented with X and V factors to enhance the recovery of *H. influenzae*, which is more likely (or was more likely before implementation of the Hib vaccine) to be isolated from children than from adults.

Frequency of bacteremic episodes versus time and frequency of collection

The frequency of bacteremic episodes is another factor to consider when determining the timing and frequency of blood collection for culture. Because bacteremia can be transient, intermittent, or continuous with respect to the presence of microorganisms in the peripheral circulation, collection of samples may depend on the type of bacteremia suspected.

In patients with transient bacteremia, organisms are immediately cleared from the peripheral system by the reticuloendothelial system, and their presence is rarely symptomatic. In suspected cases of intermittent bacteremia, patients may present clinical symptoms, especially fever, but they may not occur until after bacteria have been cleared from the bloodstream. Because these symptoms serve as the signal to obtain blood cultures, bacteremia may go undetected because of delays in obtaining blood cultures relative to the time of peak concentration of circulating bacteria. Therefore it is recommended that blood culture specimens be collected before an anticipated temperature rise to ensure maximum recovery. However, in some patients, such as those with infective endocarditis who have continuous bacteremia, the organisms are constantly released into the bloodstream and hence are likely to be isolated whenever the blood culture specimen is taken.

Rationale for multiple blood collections

Although a single set of blood culture bottles may yield the causative agent, two, three, and even four sets of blood cultures are recommended. A set consists of one bottle for recovery of aerobic organisms and a second bottle for recovery of anaerobic organisms.

There are several reasons for drawing multiple blood cultures. One study revealed that about 80% of bacteremia are discovered in the first set of blood culture specimens taken, 90% are detected if two sets of specimens are taken, and as many as 99% are detected if a third set is taken. Other studies have duplicated these results and found, in addition, that it is not so much the number of blood cultures collected that improves the detection of bacteremia but the total volume of blood that is cultured, as noted. In the past, guidelines for the collection of blood for culture stated that multiple blood cultures should be submitted when blood is collected from multiple different venipuncture sites, with 30 to 60 minutes between draws.

Although it is best to collect blood when the patient's temperature spikes or before it does, because this is when more

organisms are present in the blood, not all patients have fever when microorganisms are in the blood, and it is almost impossible to predict when fever will occur. Because organisms can be present in the blood transiently or intermittently, and there is no way to know when the organisms are present, collecting blood at the optimal time for organism recovery can be a hit-or-miss procedure. Arbitrary timing of 30- to 60-minute intervals between draws has shown no significant differences in positivity rates; therefore blood culture sets may be obtained simultaneously or consecutively as long as they are from separate venipunctures. The exception is in suspected infective endocarditis, for which 30- to 60-minute intervals are recommended to document continuous bacteremia.

According to several recent studies determining best practices for timing and blood volume collection that yield the highest percentage of positive cultures, at least 60 mL of blood should be collected in a 24-hour period, with 10 mL of blood inoculated into each bottle; three sets of two bottles each will satisfy the volume requirement and will likely result in the detection of bacteremia, if present.

Another reason for inoculating multiple bottles with blood collected from multiple separate venipunctures is to aid in the determination of whether an isolated organism is a true pathogen or a contaminant. The collection of one single sample should be strongly discouraged because the volume of blood cultured is not sufficient for detecting some infections, as noted. In addition, for example, the significance of the isolation of CoNS or other skin microbiota from one culture is hard to interpret. Their presence could represent true infection, or it could be a result of contamination. It is not until there have been repeated isolations of the same organism from multiple cultures collected from separate venipunctures that the significance can be determined.

Most blood culture systems have separate bottles for the isolation of aerobic and anaerobic bacteria, as noted earlier. This practice has been questioned because the incidence of bacteremia caused by obligate anaerobes was found to be decreasing in some studies. Some investigators have recommended that two aerobic bottles be inoculated instead to maintain the necessary volume of blood that should be collected and increase the ability to detect the aerobic and facultative anaerobes that are more commonly isolated. Additional studies into the incidence of anaerobic bacteremia, however, have shown that anaerobes are not infrequent isolates, but that their presence is difficult to predict based on analysis of clinical predictors, and that antimicrobial resistance is increasing among anaerobes, making these infections important to detect. Thus, the inoculation of separate aerobic and anaerobic bottles remains the standard recommended procedure for blood cultures, although this can differ among institutions, depending on their isolation rates of anaerobes. If the recommended amount of blood for a set of bottles is not obtained, the aerobic bottle should be inoculated first with the appropriate volume of blood; the remaining blood can be inoculated into the anaerobic bottle, rather than splitting the volume between the two bottles.

Multiple blood cultures are recommended to document bacteremia, but there is a limit to the number that should be collected. Repeated blood cultures or daily collections of blood for culture are not necessary. If at least 40 mL of blood has been cultured, and antimicrobial therapy has begun, it is best to wait for the results of the initial sets of cultures

before collecting more blood. Most organisms grow within 3 to 5 days in the continuous-monitoring systems used in most laboratories, so it is best to wait at least 3 days before collecting additional blood for culture. If no organisms grow in the blood cultures, it is more reasonable to consider other potential causes of patient symptoms, rather than culturing more blood.

Case check 36.3

The patient in the Case in Point had two sets of blood cultures drawn, a total of four bottles, which was appropriate and clinically helpful. The more sets drawn, the more likely it is that an infective agent will be recovered. The organism isolated was a CoNS, which is frequently a contaminant, but because all four bottles were positive, it was easier to determine that this was a true pathogen rather than a contaminant. If only one set had been drawn or only one bottle had been positive, it would have been difficult to make any conclusions.

Blood culture methods

Culture media used in conventional broth systems

As noted, a blood culture set typically includes a bottle designed for recovery of aerobic microorganisms and another bottle for recovery of anaerobic microorganisms. The typical aerobic culture bottle contains a nutritionally enriched medium, such as soybean casein digest broth, peptone broth, tryptic or trypticase soy broth, brain-heart infusion broth, Brucella broth, or Columbia broth base. Although anaerobic broth may contain the same types of basic media as the aerobic culture systems, 0.5% cysteine may be added to permit the growth of certain thiol-requiring organisms, and the media may be prereduced to decrease the oxidation-reduction potential to help support the growth of anaerobes. Newer anaerobic broth media, such as the BD BACTEC FX (BD Diagnostic Systems, Sparks, MD), contain blood-lysing agents that reduce the time to detection of anaerobes by lysing red blood cells (RBCs), which provides added nutrients, and WBCs, which releases phagocytized organisms. Unvented blood culture bottles generally can be used to support anaerobic organisms.

Neutralization of inhibitors

In specimens from patients receiving β -lactam antimicrobial agents, penicillinase may be added to the medium to inactivate these agents, although this is rarely done today. More commonly, commercially available automated blood culture systems generally contain antimicrobial neutralization substances such as resin or charcoal that absorb or inactivate any antimicrobial agent present in the patient's blood. The recovery of bacteria and yeasts increases with the incorporation of these neutralizing agents into the culture medium.

Anticoagulants and other additives

SPS, one of the commonly used additives, performs the following functions:

- Anticoagulation (effective at a 0.03% concentration)
- Neutralization of the bactericidal activity (i.e., complement and lysozyme) of human serum

- Prevention of phagocytosis
- Inactivation of certain antimicrobial agents (e.g., streptomycin, kanamycin, gentamicin, polymyxin B)

Despite its usefulness in blood culture media, SPS inhibits the growth of certain organisms, notably *P. anaerobius*, *N. gonorrhoeae*, *N. meningitidis*, and *Gardnerella vaginalis*. If these organisms are suspected, 1.2% gelatin added to the blood culture bottle may help neutralize the inhibitory effect of SPS.

Other anticoagulants and supplements are available for use in blood culture systems, but they are not better than SPS. Sodium amylosulfate (SAS) is a structural relative of SPS that is less effective in neutralizing serum bactericidal activity and is inhibitory to *K. pneumoniae*. Sodium citrate (0.5% to 1.0%), an anticoagulant, is inhibitory to some gram-positive cocci. Sucrose (10% to 30%) is sometimes used as an osmotic stabilizer. Sucrose is especially helpful in dealing with bacteria that have undergone cell wall damage, and the resultant hypertonicity counteracts the normal bactericidal effect of blood. Other supplements, such as the anticoagulants ammonium-potassium or sodium oxalate and EDTA, are not recommended.

Incubation conditions

Continuous-monitoring blood culture instruments, as described later, include an incubator. An incubator used to incubate agar media plates can be used to incubate blood culture media of manual systems. In automated systems, blood culture bottles are typically incubated at $35^{\circ} \pm 2^{\circ}\text{C}$ for 5 days and may be held for 7 days for manual systems. During incubation of the bottles in a continuous-monitoring blood culture system, the aerobic bottles and, in some systems, the anaerobic bottles as well may be agitated during incubation. The agitation can take the form of a rocking motion, BacT/ALERT (bioMérieux, Durham, NC) and BACTEC (BD Diagnostic Systems), or a rotary motion with the formation of a vortex, VersaTREK (TREK Diagnostic Systems, Independence, OH). Agitation of the bottles increases oxygenation of the aerobic broth, enhancing the detection of microorganisms in the bottles and decreasing the time to detect growth. The head space of the bottles contains ambient air, sometimes with increased concentration of carbon dioxide (CO_2) in the aerobic bottles. The anaerobic bottles will have an increased concentration of nitrogen (N_2) and CO_2 replacing all the oxygen (O_2) in the bottle to facilitate isolation of obligate anaerobes.

Blood culture systems

Manual systems

Although only three manual blood culture systems are currently marketed and rarely used in the United States, they are important to understand for clinical microbiologists in areas that do not have resources for or access to automated systems. They are easy to use, inexpensive, and generally adequate for the detection of common bacteria and fungi. A broth-slide system (Septi-Chek, BD Diagnostic Systems) was designed from the original biphasic (solid agar and broth combination) blood culture medium called the *Castañeda culture bottle*. Septi-Chek consists of a slide paddle containing chocolate (CHOC), MacConkey (MAC), and malt extract agars (selective for yeast and fungi) attached to the top of a standard

broth bottle. Once these bottles have been inoculated, they should be tipped daily or at least twice weekly to bathe the slide paddle with the broth culture medium, thereby allowing frequent blind subcultures without the use of needles and syringes. Bacterial growth appears as small discrete colonies or as a confluent growth on the slide paddle. Most organisms will grow within 48 hours of inoculation, but the bottles are incubated for 7 days before they are discarded and reported as negative. This system has the advantage of providing more rapid recovery of facultative anaerobic bacteria and isolated colonies for identification and susceptibility testing. There are disadvantages, however, including a slightly higher cost of materials and contamination rate, and they are labor-intensive. An additional unvented bottle is still required for adequate isolation of anaerobes.

The Oxoid Signal system (Thermo Fisher Scientific, Waltham, MA) is a manual blood culture system in which blood is inoculated into a bottle containing a liquid medium that will support the growth of aerobes, anaerobes, and microaerophiles. A clear plastic signal device is then attached to the top of the bottle. The Oxoid Signal device has a long needle that extends down into the bottle below the level of the liquid. When microorganisms grow in the bottle, they generate CO_2 , which accumulates in the head space of the bottle. The increase of gas in the atmosphere of the bottle increases pressure on the liquid, forcing it up through the needle and into the clear plastic signal device. The presence of fluid in the signal device can be seen by the microbiologist during daily inspection of the bottles and indicates bacterial growth. The fluid from the signal device can then be removed for Gram staining and plating. The inoculated Oxoid bottles are incubated at $35^{\circ} \pm 2^{\circ}\text{C}$, agitated on a shaker, for the first 24 hours. Bottles are held for a total of 7 days, with a terminal blind subculture performed and examined before the culture is reported as negative.

The lysis centrifugation method (Isolator/Isostat; Wampole Laboratories, Inverness Medical Professional Diagnostics, Princeton, NJ) has been shown to provide optimal recovery of unusual fastidious bacteria such as *Bartonella*, yeasts, filamentous and dimorphic fungi, and mycobacteria that are causing systemic infections. This method produces a concentrated sample of blood for direct inoculation onto appropriate solid agar media, as opposed to the two systems described earlier, in which blood is inoculated and incubated in a broth medium. The Isolator tube contains a mixture of saponin, propylene glycol, SPS, and EDTA. This mixture causes lysis of WBCs and RBCs, releases intracellular organisms, prevents clotting, and neutralizes complement. Microorganisms are concentrated through high-speed centrifugation (3000g for 30 minutes). The sediment containing the organisms is directly inoculated onto solid culture media that includes fungal and mycobacterial media.

Examination of blood culture bottles in a manual system

Blood culture bottles are examined macroscopically with transmitted and reflected light for evidence of turbidity, hemolysis, gas production, or bacterial colonies in or on the blood layer. If visible growth is observed, 0.25 mL of blood should be aspirated with a sterile needle and syringe. A

smear is prepared for Gram stain and then plated to a solid medium. In addition to examination of the blood culture fluid for evidence of microbial growth, the Septi-Chek paddle is examined daily for the presence of colonies; the Oxoid bottle Signal device is examined daily for the presence of fluid, indicating microbial growth in the medium. Any finding of microbial growth should be reported immediately to a health care provider.

Continuous-monitoring blood culture systems

The BACTEC system (BACTEC 460; BD Diagnostic Systems) was the first automated growth detection system for blood cultures that detected the growth of microorganisms using radiolabeled carbon (^{14}C) glucose in the broth medium. When the organism in the blood culture bottle used the ^{14}C -labeled substrate, $^{14}\text{CO}_2$ was released. The instrument monitored $^{14}\text{CO}_2$ production by aspirating gas into an ionization chamber by using sterile needles injected into the bottle. In the ionization chamber, the amount of $^{14}\text{CO}_2$ produced was measured as a growth index and compared with an established threshold level. If the patient's blood culture showed a growth index that exceeded the threshold level, the instrument sent a signal indicating that the bottle was positive. The automated radiometric blood culture system had the advantage of early detection of bacterial growth, especially of slow-growing bacterial species (e.g., *Mycobacterium tuberculosis*). The disadvantages included the high initial cost of the instrument, high contamination rate (the result of inadequate needle sterilization

between bottles), and hazards and expense associated with radioisotope disposal. The development of subsequent automated blood culture instruments kept the detection of CO_2 as an indication of microbial growth.

BACTEC 9000 series and BD FX

To eliminate radioactive isotopes as a growth detection mechanism, BD Diagnostic Systems introduced the BACTEC 9000 series. Three models are currently available—the 9240 (holds 240 bottles per module), the 9120 (holds 120 bottles per module), and the 9050 (holds 50 bottles per module). The FX holds 400 bottles per module. These are noninvasive, continuous-monitoring blood culture instruments that use fluorescence to detect CO_2 . When a bottle is placed into the instrument, a baseline reading of the sensor is taken; this reading is used as a reference for subsequent readings. When microorganisms grow in the bottle, the CO_2 that they produce is detected by a gas-permeable sensor on the bottom of each vial (see later).

CO_2 produced by an organism diffuses into the sensor, generating hydrogen ions. The increase in hydrogen ion concentration increases the fluorescence output of the sensor. Using photodetectors, the instrument measures the amount of fluorescence every 10 minutes, which corresponds to the amount of CO_2 produced by the microorganism. A computer program then interprets these data using several algorithms to determine when to flag a bottle as positive. The instrument alerts the microbiologist to the presence of a positive vial by displaying a message on the computer monitor and with an audible alarm. Figure 36.1 shows the BACTEC 9000 schematic.

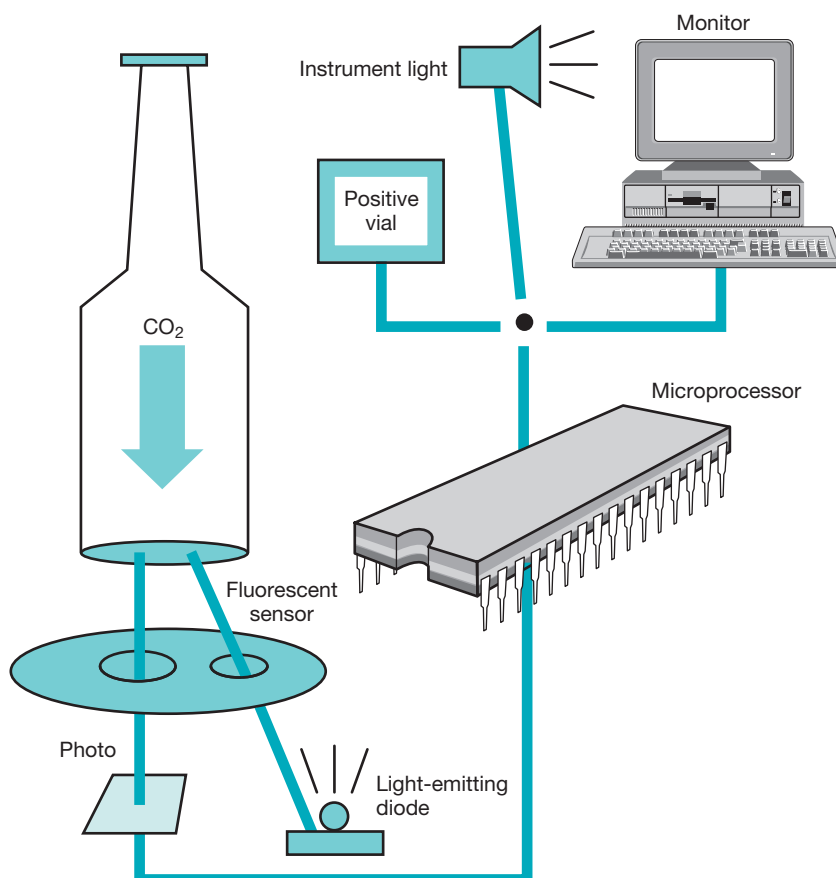


Fig. 36.1 BACTEC 9000 schematic. (Courtesy Becton, Dickinson, Sparks, Md.)

VersaTREK

The former Difco ESP system has been replaced by the VersaTREK automated detection system. VersaTREK differs from the other continuous-monitoring systems in that it detects the consumption or production of multiple gases (CO₂, hydrogen [H₂], and O₂) by organisms growing in the culture medium. These gases are detected by monitoring changes in head space pressure. The advantages to this system include the detection of different gases that may be produced or consumed by an organism present in the bottle, not just production of CO₂, and earlier detection of growth by detecting the consumption of gas as organisms enter the log phase of growth, before they start to produce CO₂. An internal computer algorithm monitors the changes in pressure, plots the pressure against time to derive a growth curve, and determines when to flag the bottle as positive.

BacT/ALERT 3D system

A fully automated, nonradiometric blood culture system, the BacT/ALERT 3D system, consists of aerobic and anaerobic bottles with pH-sensitive membranes placed in the bottom of the bottles. Microbial growth causes a release of CO₂, which changes the pH in the sensor, as indicated by a change in color from gray to yellow. The color change is measured by reflected light. The instrument measures CO₂ production colorimetrically without entering the bottles. An advantage of this system is that the changes in the color of the sensor can be detected and verified, if necessary. Another advantage of this system is that in 2003, bioMérieux released gas-impermeable plastic blood culture bottles that are safer and lighter than traditional glass bottles and do not interfere with microorganism growth or metabolism.

Recovery of other types of organisms from blood

The most common organisms isolated in one study of health-care-associated bacteremia are listed in [Table 36.2](#). All organisms listed in this table are easily recovered by traditional routine blood culture methods. Other organisms, however, can be found in blood that require special conditions to be detected. Communication with the health care provider is critical in deciding when and what extraordinary procedures should be performed.

Francisella tularensis

Francisella tularensis, the causative agent of tularemia, is best recovered from a liquid blood culture medium to which L-cysteine and glucose have been added.

Leptospira spp.

Leptospira spp. are best recovered early in the disease before onset of symptoms. Therefore blood should be collected as soon as this infection is suspected, during the first week of disease, with 1 to 3 drops of freshly drawn blood placed in 5 mL of Fletcher medium. Alternatively, 0.1 mL of heparinized blood can be inoculated onto Ellinghausen-McCullough-Johnson-Harris medium. Cultures are incubated at 30°C for 1 to 3 months in the dark and examined weekly by using dark-field microscopy. Other methods of diagnosis (polymerase

chain reaction [PCR], serology) are recommended because they are often more sensitive and timelier for detection of this organism, and treatment can be initiated more quickly, resulting in more favorable patient outcomes.

Brucella spp.

Brucella spp. require extended incubation times. When *Brucella* is suspected, conventional, manual blood culture medium (Ruiz Castañeda) should be held for up to 6 weeks at 35° ± 2°C and terminally subcultured. Automated blood culture systems show higher yields and faster recovery, but bottles should be held for 10 to 14 days. The manual lysis centrifugation system (Isolator) shows increased yields but carries the risk of exposure during manipulation and plating. Handling of suspected *Brucella* specimens and cultures should be done using special safety precautions, at least BSL 2 and preferably BSL 3 conditions, and sent to state health laboratories for confirmation and speciation. *Brucella* is usually diagnosed by serologic assays, but culture is considered the gold standard.

Nutritionally variant streptococci

The nutritionally variant streptococci (NVS) include *Abiotrophia* and *Granulicatella*. These streptococci are adequately recovered by using standard broth culture bottles because of the vitamin B₆ present in human blood. However, a pyridoxal-containing blood agar medium, addition of a pyridoxal disk to a blood agar subculture plate, or staphylococci streak is necessary for subculture to recover this group of streptococci on standard sheep blood agar, though they grow well on CHOC agar. The staphylococci streak test is performed with a confluent growth of the test organism on a blood agar plate. A single line of *S. aureus* is streaked across the middle of the plate. Following 24 hours of incubation, the plate is observed for satellitism—tiny colonies growing near the *S. aureus*.

Campylobacter spp.

Campylobacter lari, *Campylobacter fetus*, and *Campylobacter upsaliensis* can be isolated from blood. Although *Campylobacter jejuni* may grow in blood culture bottles, subcultures will show no growth unless the broth is subcultured onto selective media or enriched media, such as CHOC agar, and incubated in a microaerophilic environment at 42°C for 48 hours. A clue that the bottle contains *Campylobacter* will be the presence of curved gram-negative rods in the blood culture broth. These organisms are often difficult to find in the Gram-stained smears of the blood culture bottle; counterstaining with the darker pink basic fuchsin instead of safranin may help with visualization. An alternative stain, such as acridine orange (AO), can also be used.

Coxiella burnetii

Coxiella burnetii is the most common fastidious bacterium reported in the literature as causing endocarditis. Because *Coxiella* is an obligate intracellular pathogen, it cannot be isolated with typical blood culturing systems. It can be isolated in cell culture, but this should not be attempted unless the laboratory is equipped to handle dangerous pathogens. Instead, *Coxiella* is better detected by serology.

Bartonella spp.

Bartonella spp. are best isolated using lysis centrifugation and plating the concentrated blood onto freshly prepared media containing 5% horse or rabbit blood. Plates must be incubated for at least 3 weeks in a humid atmosphere containing elevated CO₂ levels at 35° to 37°C. Because the isolation of *Bartonella* spp. is difficult, serology and molecular methods of detection are preferred.

HACEK group of gram-negative bacilli

These bacteria (see [Chapter 18](#)) are known causes of endocarditis and will grow in blood culture bottles within 5 days. Extended incubation times and a terminal subculture are recommended only if blood cultures are negative at 5 days and there is a high clinical index of suspicion for these organisms.

Mycobacteria from blood

Using special media, *Mycobacterium* spp. can be detected with the automated blood culture systems discussed earlier. Medium in the bottles contains a lysing agent and enrichment (Middlebrook based) designed for long incubation periods, up to 6 weeks. The Isolator system can also be used to culture blood for mycobacteria. Plated selective media are held for 6 to 8 weeks before mycobacteria can be ruled out.

Fungemia

The presence of fungi in the blood is termed **fungemia**. *Candida* spp. are isolated almost as frequently as are gram-negative bacilli from blood in some institutions. Yeasts grow in the continuous-monitoring blood culture systems. The best way to recover filamentous or dimorphic fungi in blood, however, is to use the Isolator system with fungal agar plates, which are usually held for 4 weeks. Yeasts and dimorphic fungi are the most common fungi isolated from blood.

Viremia

Viremia, the presence of viruses in blood, is determined by serology or molecular-based detection systems. Viruses that can be detected from blood include cytomegalovirus, Epstein-Barr virus, adenoviruses, and enteroviruses.

Contamination in blood cultures

A contamination rate of 1% to 3%, although not desirable, can be expected, with CoNS, *Micrococcus* spp., diphtheroids, and *C. acnes* as common contaminants. Any organism cultured from two or more blood culture bottles, however, should not be assumed to be a contaminant. All of the organisms mentioned can also be responsible for bacterial endocarditis. Contamination rates should be monitored; if they increase above 3%, an examination of phlebotomy practices should be performed along with reeducation of phlebotomists and nurses with regard to appropriate methods for collecting blood for culture.

Other sources of contamination causing pseudobacteremia include IV catheters as well as various skin disinfectant solutions. Benzalkonium chloride has been demonstrated to be occasionally contaminated with *Burkholderia cepacia* and

Enterobacter spp. Alcohol or iodine as a skin disinfectant has been preferred. However, contamination of certain iodine solutions, such as povidone-iodine, with *B. cepacia* has also been reported. Contamination with *Serratia marcescens* and *Moraxella* spp. has been reported in some evacuated blood collection tubes.

In the past, contamination was more clear-cut. The media and volume of blood used now are more sensitive and grow more contaminants. It can be difficult to determine the difference between a true pathogen and contamination. As the volume of blood increases, the number of positive blood culture results increases. Microbiologists cannot make this determination (true pathogen or contaminant) in the laboratory; health care provider input and patient history are needed. Criteria that can be used to determine whether an isolate from a blood culture is a pathogen or contaminant include the following:

- Identity of the microorganism isolated. *S. aureus*, *E. coli*, and other members of the Enterobacterales; *S. pneumoniae*; *P. aeruginosa*; and *C. albicans* almost always indicate true infection, whereas CoNS, diphtheroids, and other typical skin microbiota should be questioned.
- More than one blood culture bottle growing the same organism usually indicates a significant isolate, whereas growth of an organism, especially one associated with skin microbiota, in a single bottle when multiple sets of blood cultures are obtained usually indicates that the isolate is a contaminant.
- Isolation of the same organism from the blood and from a normally sterile site in the same patient usually indicates that the organism isolated from the blood is a pathogen.

Rapid identification of microorganisms growing in blood cultures

Automated continuous-monitoring blood culture systems have taken the labor out of examining blood culture bottles for the presence of microbial growth, but these systems can determine only that something is growing in the bottle. The microbiologist must remove a flagged bottle from the instrument and process the fluid to determine whether a viable microorganism is present and identify it. A small amount of fluid is removed from the bottle and used to make a smear to be Gram stained and plated onto appropriate media, depending on microscopic morphology of the organisms observed on the Gram-stained smear. All initial procedures from the blood culture bottle should be performed in a biological safety cabinet using standard safety precautions.

An anaerobic blood agar plate should be subcultured from every positive bottle, aerobic or anaerobic, because anaerobes sometimes grow from aerobic bottles, and vice versa. The Gram-stained smear can be examined quickly, and the health care provider should be notified immediately of the positive blood culture result along with the morphology of the organism seen (e.g., gram-positive cocci, gram-negative rods, yeast). Definitive identification and susceptibility results are typically not available until isolated colonies grow on the subcultured plates, which usually takes 24 to 48 hours. During this time, the patient is given empiric antimicrobial therapy that may or may not be optimally effective for the isolate. The sooner the patient is treated with an effective antimicrobial

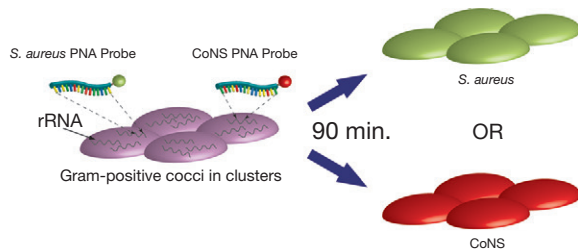


Fig. 36.2 Peptide nucleic acid (PNA) fluorescent in situ hybridization. Based on initial Gram stain of the positive blood culture, this method can identify commonly isolated organisms within 90 minutes. This example shows the differentiation of *Staphylococcus aureus* and coagulase-negative staphylococci (CoNS). *rRNA*, Ribosomal ribonucleic acid.

agent, the more favorable the outcome. To decrease the time it takes to identify a microorganism growing in a blood culture, several rapid methods are available.

Conventional rapid diagnostic tests

Biochemical and enzymatic tests and stains that can be performed directly from blood culture bottles include direct tube coagulase (to differentiate suspected *S. aureus* from CoNS), thermonuclease (a heat-stable DNase produced by *S. aureus*), oxidase, bile solubility and Quellung capsule stain (for suspected *S. pneumoniae*), and modified Kinyoun stain (AFB/*Nocardia*). These are presumptive, not definitive tests but can provide the health care provider with preliminary information. With the development of rapid and accurate molecular identification directly from blood bottles, these tests are rarely performed currently.

Fluorescence in situ hybridization

Fluorescence in situ hybridization targets ribosomal ribonucleic acid (rRNA) in an organism by using an oligonucleotide or peptide nucleic acid (PNA) probe with a fluorescent label. Although this is a molecular method, there is no amplification of nucleic acid involved. The procedure takes 1.5 to 3 hours to perform; the sensitivity of species-specific probes in one study was reported to be 97%, with 95% specificity. Different probes are available that target common organisms isolated from blood cultures, such as *S. aureus*, *Enterococcus* spp., and *Candida* spp. (Fig. 36.2).

Nucleic acid amplification methods

Several nucleic acid amplification tests (NAATs), using PCR to directly identify microorganisms growing in a blood culture, have been approved by the U.S. Food and Drug Administration (FDA) and are available. The Verigene system (Nanosphere, Northbrook, IL) amplifies nucleic acid targets that are then detected by hybridization of oligonucleotides bound to nanosphere particles with a silver staining process (Fig. 36.3). The FilmArray system (BioFire Diagnostics, Salt Lake City, UT) involves an initial nucleic acid extraction and purification followed by PCR amplification in a two-step process. The Verigene, FilmArray, and ePlex (GenMark Diagnostics, Carlsbad, CA) systems are multiplex assays that can identify a group of gram-positive and gram-negative organisms and yeasts, as well as several resistance genes, within 1.5 to a few hours. The GeneXpert system (Cepheid,



Fig. 36.3 Verigene test cartridge (Nanosphere, Northbrook, IL). Nucleic acid amplification testing can identify commonly isolated organisms and some species with resistance genes within 2 to 4 hours. Isolate from the blood culture bottle is inoculated into wells.

Sunnyvale, CA) also offers assays that detect resistance genes and is evaluating a molecular assay (XPert MTB/RIF) to detect *M. tuberculosis* directly from blood. Clinical trials have shown greater than 95% concordance for identification and drug resistance compared with conventional culture methods, resulting in a great reduction in time to reporting and appropriate treatment, costs, and hospital stays for patients. A disadvantage is that these methods do not perform well in the case of polymicrobial infections, and there is some ambiguity in the identification of certain groups of organisms. Therefore it is important to note that these methods do not replace conventional blood cultures but are used in conjunction with them.

Matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry

Matrix-assisted laser desorption/ionization–time-of-flight (MALDI-TOF) mass spectrometry identifies a wide variety of organisms, including fungi, fastidious bacteria, and anaerobes, in a matter of minutes, and it is routinely used in many modern microbiology laboratories. The Vitek MS (bioMérieux, Inc.) and the Microflex (Bruker, Billerica, MA) are instruments that use this technology (Fig. 36.4). The organisms must first be subcultured from the blood and isolated in pure culture. The FDA has not approved direct detection from blood, and there is currently no ability to determine antimicrobial susceptibility by using this method.

Biomarkers

Sepsis is the most common cause of admissions and death in intensive care units, and the rates are increasing. Sepsis is difficult to diagnose using clinical signs and symptoms. Some of the classic indicators of sepsis (i.e., elevated WBC count, fever, elevated CRP level, and hypotension) can be absent during sepsis. In addition, these indicators can occur in other systemic inflammatory conditions, including trauma and surgery, as well as from a response to various drugs and blood product transfusions. Although blood cultures are considered the gold standard, they are positive in only about one third of sepsis cases and generally require 3 to 5 days for a positive result to be obtained. Rapid accurate diagnosis of sepsis is



Fig. 36.4 Microflex matrix-assisted laser desorption/ionization–time-of-flight instrument (Bruker, Billerica, MA). This is a 48- to 96-organism per test plate that can identify isolates within 15 to 20 minutes. The organisms must first be grown in pure culture.

BOX 36.2 Biomarkers for sepsis

Some of the more commonly used diagnostic and/or prognostic biomarkers for sepsis are as follows:

- (1-3)- β -D-Glucan^a
- Lactate
- Procalcitonin
- C-reactive protein
- D-dimer
- Endocan
- Inducible protein 10
- Group IV phospholipase A2 type II
- Neutrophil gelatinase-associated lipocalin
- Lipopolysaccharide-binding protein
- Macrophage migration inhibitory factor
- Mature adrenomedullin
- Mer receptor
- Midregional proadrenomedullin
- Natriuretic peptides
- Copeptin
- Thrombopoietin
- Soluble triggering receptor expressed on myeloid cells 1
- Soluble urokinase-type plasminogen activator

^aMarker for invasive fungal infection.

critical for appropriate lifesaving treatment, and it is important to rule out sepsis to prevent unnecessary treatments. For these reasons, scientists have been looking for biomarkers to aid in the diagnosis. A biomarker is defined as an objectively measured characteristic that indicates a normal biological or pathogenic process, or pharmacologic response to therapy.

Over 175 different biomarkers for sepsis, with diagnostic and/or prognostic value, have been reported in the literature (Box 36.2). The goal of such a marker is to distinguish between sepsis and any other SIRS. Procalcitonin, a peptide released in response to proinflammatory stimuli, is regarded

as a potential biomarker for sepsis. In a large meta-analysis, it had a reported sensitivity of 77%. If the test were used alone to diagnose sepsis, 23% of the patients with a sepsis would have a negative result and would not receive antimicrobial therapy. The reported specificity was 79%; that is, 21% of the patients without sepsis would test positive and would undergo unnecessary treatment. It is clearly not an ideal test in several clinical situations, and many health care providers are reluctant to make treatment decisions based solely on procalcitonin test results.

Because individual biomarkers seem to have poor predictive ability in diagnosing sepsis, scientists and health care providers have turned to combinations of biomarkers. For example, serum lactate (lactic acid) has been found to be helpful in indicating severe sepsis, particularly when used with CRP or procalcitonin levels. Other studies have reported on numerous combinations with as many as six different biomarkers. The combination assays had a better predictive value than any one biomarker. Currently, health care providers use clinical signs and symptoms along with the results of a select number of biomarkers to guide therapy. Research is still needed to determine which markers and which combinations provide the best diagnostic value.

Treatment

Sepsis being such a complex disorder, it is expected that many therapeutic options are needed to alleviate this devastating clinical event. Several treatment modalities have been tried, but some are not always successful.

Antimicrobial therapy

Antimicrobial agents remain the mainstay of treatment of bacteremia. Because of the potentially devastating consequences of untreated bacteremia, therapy is frequently instituted empirically on the basis of clinical signs and symptoms before bacteremia has been confirmed by a positive blood culture or before the causative agent has been definitively identified. The choice of initial therapy is based on the likely identity of the infecting microorganism (according to the patient's clinical syndrome), presence of comorbid conditions that might affect the risk of infection with particular bacterial species (e.g., cancer), patient's immune status, recent environmental exposures (e.g., hospitalization that might have resulted in colonization by resistant organisms), and prior antimicrobial therapy, which might have selected for resistant pathogens. Broad-spectrum antimicrobial agents are frequently used for initial empiric therapy, and a combination of agents may be used to ensure coverage of several possible pathogens.

After identification of organisms cultured from blood and determination of antimicrobial susceptibility, initial therapy may be more narrowly targeted, thus allowing use of the most effective agent against the responsible pathogen while minimizing the potential for adverse reactions and emergence of antimicrobial resistance. For bacteremia caused by some pathogens (e.g., *E. faecium*, *P. aeruginosa*), a cell wall-active agent, such as a β -lactam, is combined with an aminoglycoside, resulting in a synergistic antimicrobial effect and improved clinical outcome. Along with antimicrobial therapy, adjunctive measures, such as drainage of infected

fluid and removal of an infected intravascular catheter, may be essential to achieving cure of the infection. Treatment of comorbid conditions, such as diabetes, is helpful in gaining control of infection. Finally, measures aimed at restoring immunocompetence—such as administration of cytokines that increase the number of circulating neutrophils in patients who are immunosuppressed because of cancer chemotherapy—are critical in complementing antimicrobial therapy.

Antisepsis therapy

Because of the high mortality of septic shock, whether or not accompanied by bacteremia, a number of therapies aimed at blocking the cascade of events that result in sepsis, shock, and death have been studied and evaluated. These therapies described here are invariably used in combination with antimicrobial agents. Unfortunately, even with treatment, 30% to 50% of patients with sepsis die, usually because of underlying illnesses in addition to their sepsis.

Physiologic support

Resuscitation with IV fluids to maintain tissue perfusion is a fundamental method for management of the patient with sepsis. In patients with septic shock who do not respond to fluid support, pressor agents may be used to ensure an adequate blood pressure. Along with fluids, respiratory therapy with oxygen is used to support patients with sepsis.

Anticoagulation agents

Because the consequences of bacteremia and sepsis may include activation of the coagulation cascade with resultant DIC and decreased tissue perfusion, the use of anticoagulants has been studied for the treatment of sepsis. One agent, drotrecogin alfa, also known as *activated protein C*, has been shown in clinical trials to decrease mortality in patients with septic shock. Drotrecogin alfa inhibits factors Va and VIIIa of the coagulation cascade, thereby inhibiting coagulation. Drotrecogin alfa may also decrease chemotaxis of WBCs by interfering with the interaction between the leukocytes and endothelium of blood vessels. Because of the potential bleeding complications, this therapy is restricted to use in patients who are at the highest risk of death. It is not effective for less severely ill patients. Other anticoagulant therapies, such as antithrombin III therapy, have not been effective in the treatment of sepsis.

Glucocorticoids

Because of their potent anti-inflammatory action, glucocorticoids have long been of interest in the treatment of sepsis. Although early studies of high-dose glucocorticoids showed benefit in septic patients, a randomized controlled trial in the late 1980s showed no advantages to the use of glucocorticoids over placebo in severely ill patients with sepsis, and their use was abandoned. More recently however, the use of glucocorticoids has made a cautious comeback based on controlled trials of low doses of these agents to treat adrenal insufficiency associated with sepsis rather than to block inflammation.

Case check 36.4

The bacterium isolated from the patient in the Case in Point was a CoNS. Initially, the patient was given broad-spectrum antimicrobial agents, based on the initial report of gram-positive cocci in clusters seen on Gram-stained smear. However, once the organism was identified and susceptibility testing performed, treatment was changed to more appropriate antimicrobials to minimize the potential for adverse side effects or emergence of resistance.

Anticytokine therapies

A large number of investigational agents aimed at blocking the action of tumor necrosis factor and other cytokine mediators of sepsis have been studied in the treatment of sepsis. Despite an apparently sound theoretic rationale and promising results in animal models, these therapies have not proven effective in clinical trials.

Prevention

Prevention of community-acquired bacteremia has been aided by immunizations. Pneumococcal and Hib vaccination have been effective in preventing invasive infections caused by *S. pneumoniae* and *H. influenzae*, respectively, and vaccination for viral pathogens (e.g., influenza virus, varicella virus) has helped lower the incidence of invasive secondary infections caused by bacterial pathogens, such as *S. aureus* and *S. pyogenes*. In the hospital setting, prevention of healthcare-associated bacteremia revolves around minimizing iatrogenic infections from indwelling intravascular catheters and other invasive devices by following recommended infection control practices. The use of antimicrobial-coated central venous catheters has been shown to decrease rates of CRBSIs. Some hospitals currently screen and treat patients colonized by some organisms, such as MRSA, VRE, and resistant gram-negative rods, before surgery to lessen the risk of these patients developing bacteremia after surgery.

POINTS TO REMEMBER

- Patients who are immunocompromised are at greatest risk for bacteremia and the systemic complications that may follow.
- Most bacteremia cases in the United States are caused by gram-positive organisms, although gram-negative organisms are increasing and represent a large proportion of cases.
- Fungal agents are significant causes of bloodstream infections (fungemia).
- Time of collection, frequency of obtaining, and volume of blood for culture are critical components for optimal recovery of microorganisms.
- Instrumentation for laboratory diagnosis of bacteremia continues to become more rapid and sensitive for the detection of organisms present in blood cultures.
- Rapid diagnostic tests for the identification of organisms in blood are widely available.
- A combination of biomarker test results along with clinical signs and symptoms might provide a rapid and accurate diagnosis of sepsis.
- Antimicrobial agents remain the mainstay of treatment of bacteremia.

LEARNING ASSESSMENT QUESTIONS

1. Which form of bacteremia is demonstrated in the Case in Point?
2. What condition has placed the patient at an increased risk for bacteremia?
3. Which other risk factors favor bacteremic episodes in certain patient populations?
4. What are the most common sources of bacteremia?
5. Which bacterial organisms in pneumonias produce concurrent bacteremia?
6. What are the major consequences of bacteremia?
7. What are treatment methods for bacteremia?
8. Who is at risk for polymicrobial bacteremia?
9. Why is it important to keep the ratio of blood to culture medium to 1:10?
10. How many sets of blood cultures should be collected, and how often should they be collected for maximum recovery of infecting agents?

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Urinary tract infections

Lindsey E. Nielsen

OUTLINE

Introduction, 923

Anatomic considerations, 923

Epidemiology and risk factors, 925

Age, 925

Pregnancy, 927

Renal transplantation, 927

Bladder catheterization, 927

Sexually transmitted infections, 927

Clinical signs and symptoms, 927

Causes of urinary tract infections, 928

Pathogenesis, 928

Causative agents, 929

Laboratory diagnosis, 931

Significance of colony counts: historic background, 931

Specimen collection and transport, 932

Additives, 934

Microbial detection, 934

Specimen screening: rapid nonculture methods, 934

Molecular screening and identification methods, 935

Rejection criteria, 936

Culture for causative agents of urinary tract infections, 936

Interpretation of results, 937

Susceptibility reporting, 937

Urinary tract infection antibiograms, 938

Bibliography, 940

- Describe the laboratory diagnosis of UTIs, including specimen collection, screening methods, and interpretation of colony counts.
- Correlate the urinalysis results with the bacterial colony count and signs and symptoms presented by the patient.
- Interpret urine culture results given patient signs and symptoms.
- Justify performing a urine leukocyte count and consider the patient's clinical presentation when interpreting a urine culture.

KEY TERMS

Acute urethral syndrome

Asymptomatic bacteriuria (ASB)

Bacteriuria

Cervicitis

Cystitis

Dysuria

Prostatitis

Pyelonephritis

Pyuria

Ureteritis

Urethritis

Urinary tract infection (UTI)

Case in point

A 26-year-old female patient presented to the urgent health clinic with complaints of urinary frequency, dysuria, hematuria, and severe lower back pain. She was febrile and slightly tachycardiac but otherwise unremarkable. She had previous urinary tract infections (UTIs) 3 and 7 months before the current presentation, which were treated initially with ampicillin but went unresolved until a combination of nitrofurantoin and doxycycline were used. Given her past medical history, complaints of back pain, and clinical findings, she was sent to the local hospital for further observation. While at the hospital, she became delirious, and her blood pressure decreased. A sepsis and urine workup was initiated. A complete blood count showed elevated white blood cells, and a urine dipstick was positive for blood and a pH of 6.5. A microscopic analysis revealed bacteria without casts. Bacterial culture was performed by plating the urine to sheep blood and MacConkey agar plates. Growth of 100,000 colony-forming units (CFU) per mL was observed on sheep blood agar only. Gram-stained smear of the colonies revealed gram-positive cocci in clusters. The final identification

OBJECTIVES

After reading and studying this chapter, you should be able to:

- Discuss various infections that occur in the urinary system.
- Associate the clinical signs, symptoms, and parameters with each of the disease manifestations.
- Identify the epidemiology and risk factors associated with the development of a urinary tract infection (UTI).
- Identify the organisms associated with UTIs.

was *Staphylococcus aureus*, and blood cultures were flagged as positive the same day with the same organism.

Issues to consider

After reading the patient's case history, consider:

- The difference between a single-episode UTI and recurrent UTIs
- Different ways patients present with a UTI
- The value of urinalysis in diagnosing UTI
- The value of obtaining a blood culture in suspected pyelonephritis cases
- The common causative agents of UTIs

Introduction

Urinary tract infections (UTIs) are among the most common bacterial infections affecting humans, accounting for up to 7 million episodes of **cystitis** and 250,000 episodes of **pyelonephritis** infections in the United States annually. Accurately assessing the incidence of UTIs is difficult because they are generally not reportable diseases. Diagnosis depends on patient signs and symptoms as well as urinalysis and urine culture results. The most common presentation is presence of blood in the urine (**hematuria**), pain during urination (**dysuria**), and increased urinary frequency. In the outpatient setting, however, diagnosis is usually made without the latter. UTIs occur more frequently in women than in men, with up to 50% of all women experiencing a UTI during their lifetime. Those at risk for UTI include older adults, pregnant women, patients who have had renal transplantation, patients with spinal cord injuries, patients with bladder catheters, and patients with genitourinary (GU) tract abnormalities.

There are two clinical schemas for classifying UTIs: (1) single versus recurrent episode and (2) complicated versus uncomplicated episode. A single-episode UTI occurs once and does not recur. Patients with chronic or recurrent UTIs have repeated episodes of urinary infections caused by bacteria (**bacteriuria**), with or without clinical manifestations. These episodes are arbitrarily divided into relapse and reinfection. The former involves the same organism and implies a focus of infection in the renal or prostatic parenchyma; the latter implies a different organism and is usually limited to the bladder. In the clinical history of a patient, a general rule is that prior UTIs within 6 months or less from the current episode are highly likely to involve the same organism. This correlation is helpful in deciding empiric treatment and possibly determining if the source of the infection occurs from bacteria being introduced into the system via a urethral or a GU abnormality or if the infectious agent is present elsewhere in the body and is being "seeded" into the urinary tract.

Case check 37.1

A single-episode UTI occurs once, with no repeated episodes. These cases are usually not associated with GU abnormalities. Recurrent UTIs occur as repeated episodes of bacteriuria, with or without clinical manifestations. They can occur as relapse when the same infectious agent is identified or as reinfection with different organisms.

Uncomplicated UTIs occur primarily in sexually active young women and adults with normal GU tracts and no prior instrumentation; these infections are usually caused by antimicrobial-susceptible bacteria. Complicated UTIs occur in individuals who have one or more structural or functional GU abnormalities or have indwelling catheters and whose conditions cannot be controlled with therapy.

Bacteriuria, which can be symptomatic or asymptomatic, is the presence of bacteria in urine. Asymptomatic bacteriuria (ASB) is the presence of bacteria in urine in significant quantities, but without GU signs or symptoms of infection. ASB requires treatment only in some populations, such as pregnant women and patients about to undergo instrumentation of the GU tract. Disease occurs when the multiplication of organisms in the urinary tract interferes with the normal function of the involved organ. It is important to remember that infection is defined by clinical parameters and urinalysis, not solely by quantitation or identification of microbes. In fact, as more organisms are discovered, there may be several fastidious or unculturable microbes that cause ASB and UTIs.

Anatomic considerations

The urinary system includes the kidneys, where urine is formed as a plasma filtrate; the ureters, which drain urine to the bladder; the bladder; and the urethra, which excretes urine from the bladder (Fig. 37.1). In addition, the prostate gland in males is considered part of the urinary tract. The only other significant difference between the male and female urinary tract is the length of the urethra—about 4 cm in women and 20 cm in men. Because most UTIs result from microorganisms ascending the urethra, women are at greater risk for UTIs than men. Except for the urethral mucosa and the renal medulla, which appear to be relatively more susceptible to infection, the normal urinary tract is resistant to colonization and subsequent infection by bacteria. The urinary tract efficiently and rapidly eliminates virulent and avirulent microorganisms. The urinary tract is normally regarded as a sterile site; however, the urethra can be transiently colonized by microorganisms.

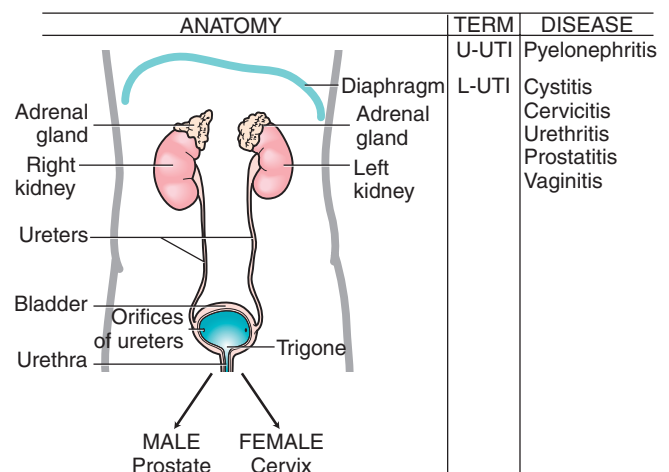


Fig. 37.1 Anatomy of the urinary tract, with corresponding terms and diseases. L-UTI, Lower urinary tract infection; U-UTI, upper urinary tract infection.

Although urine is frequently considered a good medium for bacterial growth, the extremely high urine osmolarity and low pH inhibit the growth of many uropathogens and almost all normal urethral microbiota. In addition, very dilute urine fails to support the growth of most bacterial species. Urine from men is more inhibitory than urine from women because of the bactericidal effect of prostatic fluids in the urine of men as well as the difference in pH and osmolarity, leading to another reason that women have a higher incidence of UTIs.

Factors such as high ammonia concentration, hyperosmolarity, lowered pH, and sluggish blood flow in the renal medulla can contribute to reduced leukocyte chemotaxis and bactericidal activity of WBCs, resulting in lowered resistance to infection. Urine has also been shown to inhibit the migrating, adhering, activation, and killing functions of polymorphonuclear cells. However, the presence of acid-labile mucopolysaccharides that inhibit bacterial adherence along with the flushing action of the bladder provide additional defensive mechanisms along the lower urinary tract

that overcome these immunosuppressive effects. In certain cases, such as postsurgical improper draining of a catheter, anatomic abnormalities, or calculi formation (kidney stones), urinary retention may be a significant contributing factor to UTI development. Table 37.1 lists the comparative physiologic parameters of urine for healthy individuals and those with cystitis and pyelonephritis.

In most cases, the pathogenesis and course of UTIs are determined by the anatomy of the organs involved. UTIs can be distinguished by location into upper and lower UTIs. Upper UTIs (U-UTIs) involve the renal parenchyma (**pyelonephritis**) or the ureters (**ureteritis**). Lower UTIs (L-UTIs) involve the bladder (**cystitis**), the urethra (**urethritis**), and in males, the prostate (**prostatitis**). Urethritis and cystitis are the most common types of UTIs. The heterogeneity of disease presentation, management, and prognosis is reflected in the terminology of UTIs. Box 37.1 lists terms and definitions frequently used in connection with UTIs. Each has specific criteria and must be used appropriately.

Table 37.1 Comparative parameters for urine in control individuals and patients with urinary tract infections

Parameter	Ranges		
	Reference value	Abnormal	
		Cystitis	Pyelonephritis
Urine dipstick			
Specific gravity	1.001–1.035		
pH range	4.7–8.0 (6.0 average)		
Protein	Negative to trace		Increased
WBC esterase	Negative	Positive	Positive
Nitrite	Negative	Positive	Positive
Blood	Negative	Variable	Positive
Microscopic			
WBCs			
Male	0–3/hpf	Variable	Elevated to greatly increased
Female/child	0–5/hpf	Variable	Elevated to greatly increased
RBCs	0–2/hpf	Variable	Variable to greatly increased
Epithelial cells			
Squamous	1–5/hpf		Variable/hpf
Renal	0–1/hpf		Variable/hpf
Transitional	0–2/hpf		Variable/hpf
Crystals	Variable	Negative	Negative
Mucus	Variable	Negative	Negative
Casts			
Hyaline	0–2/lpf		
Granular	0–1/lpf		
WBCs	Negative	Negative	Positive
Microorganisms			
Bacteria	Less than 1/hpf	Variable	Positive
Yeast	Negative	Variable	Variable
Trichomonas spp.	Negative	Variable	Negative
hpf, High-power field; lpf, low-power field; RBCs, red blood cells; WBCs, white blood cells.			

hpf, High-power field; lpf, low-power field; RBCs, red blood cells; WBCs, white blood cells.

BOX 37.1 Terms and abbreviations commonly used for urinary tract conditions

Acute urethral syndrome. Also known as *abacterial cystitis*, includes urinary frequency, dysuria, and pyuria. It is sometimes defined as the presence of more than 8 leukocytes/ μ L in uncentrifuged urine or approximately 2 to 5 leukocytes per high-power field present in centrifuged urine sediment accompanied by urine cultures negative for bacteria.

Bacteriuria. The presence of detectable bacteria in the urine. Patients may be symptomatic or asymptomatic (e.g., geriatric or pregnant patients).

Cervicitis. Inflammation of the cervix; it may present as acute or chronic. Causative agents include sexually transmitted organisms, such as *Neisseria gonorrhoeae* and *Chlamydia trachomatis*. Symptoms include dysuria, urinary urgency, vaginal discharge, and low back pain.

Cystitis. Inflammation of the bladder, presenting as dysuria and urinary frequency and urgency. It is often caused by gram-negative bacilli, such as *Escherichia coli*, *Proteus* spp., and *Klebsiella* spp. It occurs more frequently in women than in men. It can also be caused by medication and certain viruses, such as the adenovirus.

Dysuria. Pain during urination. Often felt as a burning sensation, but may present differently for each person.

Hematuria. Presence of blood in the urine. This can be visible or may be only detected on urine dipstick or microscopic examination. More common in upper UTIs than in lower UTIs.

Lower urinary tract infection (L-UTI). A GU tract infection limited to the urethra (urethritis), bladder (cystitis), and in males, the prostate (prostatitis). These infections generally appear in adults with dysuria, increased urinary frequency and urgency, and occasionally suprapubic tenderness.

Prostatitis. A GU infection in males that involves the prostate; fever is often present.

Pyelonephritis. Infection in the kidney. This is often caused by infection in the lower tract ascending to the kidney. Symptoms include fever, chills, nausea, vomiting, lower back tenderness, and dysuria. It can be due to hematogenous spread by bacteremia.

Upper urinary tract infection (U-UTI). A GU tract infection limited to the renal parenchyma (pyelonephritis) or the ureters (ureteritis). It is often accompanied by L-UTI symptoms in addition to costovertebral flank pain or tenderness and fever. At times, L-UTI precedes the appearance of fever and U-UTI by 24 to 48 hours.

Urethritis. Inflammation of the urethra, presenting as dysuria and discharge. Causative agents include *N. gonorrhoeae* and *C. trachomatis*. Other causes include trauma, allergies, or chemical factors.

Urinary tract infection (UTI). A spectrum of diseases caused by microbial invasion of the GU tract that extends from the renal cortex of the kidney to the urethral meatus (see Fig. 37.1).

pregnancy as an adult. This necessitates extensive evaluation to identify underlying functional or anatomic abnormalities. During the neonatal period, about 1% of all infants have bacteria in their urine. The incidence is higher in boys, and bacteremia often is present. A meta-analysis from children across Europe showed that during childhood, especially in the first 12 months of life, a 5% rate of UTIs occurs. The rate of UTIs in neonates 2 weeks of age and younger is higher if in the intensive care unit (ICU) and can correlate with the presence of otherwise unexplained jaundice and sepsis. In addition, autopsies have shown a predominance of infection in infant boys with pyelonephritis.

Strong evidence exists indicating that circumcision may confer a protective effect against UTIs in male infants. Non-circumcised infants younger than 6 months of age were shown to have a 12-fold increased risk of UTI compared with circumcised cohorts. Among preschool-age children, girls develop UTIs more often compared with boys, and infection is frequently associated with severe congenital abnormalities. These infections often do not manifest any symptoms. It is believed that most of the renal damage caused by a UTI occurs in this age group. Among school-age girls, in whom UTIs go into long-term remission spontaneously or through antimicrobial therapy, many develop symptomatic infection after they engage in sexual activity, and these infections occur at a far higher rate than in the general population. Thus the presence of bacteria in urine in childhood defines a population at higher risk for the development of UTIs in adulthood.

Adults to 65 years of age

From adulthood to 65 years of age, the incidence of UTIs in men is extremely low. When infections do occur, they often are associated with anatomic abnormalities or prostatic disease and consequent instrumentation, such as catheterization. Among women in this age group, however, as many as 20% experience a UTI with associated symptoms. Women without catheters, and who are sexually active, tend to experience more UTIs than those practicing abstinence.

Geriatric population

The diagnosis and management of UTIs in the geriatric population can be challenging. Older adults frequently have an atypical clinical presentation, including delirium, fever, hypothermia, or failure to thrive. Geriatric patients also have more comorbidities (two or more unrelated diseases or coexisting medical problems) and an increased risk of drug interactions.

In patients older than 65 years of age, the incidence of UTIs increases dramatically for both sexes, but with a progressive decrease in the female-to-male ratio. The increased incidence of UTIs in men arises from obstructive uropathologic conditions caused by the prostate and from the loss of the bactericidal activity of prostatic secretions. In women, bladder prolapse contributes to the occurrence of infection, as does soiling of the perineum from fecal incontinence in women with dementia and estrogen deficits causing changes to the vaginal biota. In both men and women, neuromuscular disease, decreased relative mobility, and increased instrumentation and bladder catheterization are

Epidemiology and risk factors

Age

Children

UTI in children is associated with great morbidity and long-term medical problems, including impaired renal function, hypertension, end-stage renal disease, and complications of

contributing factors. UTI is a common cause of bacteremia in older adults.

The time course of UTIs is shown in Fig. 37.2. The epidemiology of UTIs is influenced by the pathophysiology of the infection and by other factors, such as the virulence of the infecting organisms and their inherent mechanisms of pathogenicity, the person's immune status, and other selective external pressures. Box 37.2 lists well-defined microbial virulence factors, and Table 37.2 presents nonspecific and GU-specific factors that affect host defense and the immune system's ability, humoral and cellular, to resist infection. The dynamic interaction that exists among these factors is continually changing.

The severity of infection cannot be defined by the type of invading organism or by colony counts alone; the complete clinical situation must be assessed. Predisposing factors that may affect a person's risk of developing a UTI include presence of an indwelling catheter, or intermittent catheterization; urinary tract instrumentation, manipulation, or structural abnormality; and underlying disorders or comorbidities, such as diabetes mellitus. Other underlying risk factors include sexual activity in younger women and incontinence in the geriatric age group. Nonspecific factors that can significantly enhance the risk for infection directly or indirectly, include smoking and the use of birth control pills, alcohol, or antimicrobial agents. Common urinary pathogens in older adult populations include *S. aureus*, enterococci, and *Proteus mirabilis*, particularly in patients with catheter-associated urinary tract infections (CAUTIs).

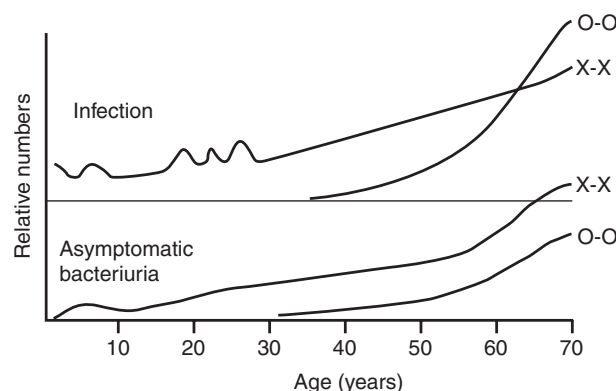


Fig. 37.2 Frequency of urinary tract infection over time. O-O, Males; X-X, females.

BOX 37.2 Microbial virulence factors

- Adherence (bacterial adhesions)
- Biofilms
- Capsular polysaccharides
- Hemolysins
- Lipopolysaccharides
- Mobility (flagella and chemotaxis responses)
- Toxin production

Case check 37.2

Patients can present with UTI differently based on age, location of infection, and underlying conditions or comorbidities. Children younger than 2 years may present with failure to thrive, vomiting, and lethargy. Children older than 2 years may complain of dysuria and abdominal pain. Adults may complain of dysuria, urgency, frequency, and suprapubic pain. They might have fever, nausea and vomiting, flank pain, and pyelonephritis. Older patients can have delirium, weakness, and failure to thrive; transplant patients may have pain at the graft sites as well as changes in mentation.

Institutionalized care

Hospitalized patients and those residing in long-term care facilities develop UTIs more often compared with outpatients. The generally ill condition of patients in health care institutions, higher probability of urinary tract instrumentation, and higher incidence of GU tract anatomic or functional abnormalities are major contributors to this difference.

ASB is a common and usually benign condition in this population. Neurogenic bladder and incontinence increase the frequency of ASB. It can be difficult to distinguish ASB from a symptomatic infection. In one study of nursing home patients with fever, a UTI was the cause in only 10%. However, most patients had a positive urine culture result because of the underlying ASB. A serious cause of fever, such as pneumonia, bacteremia, or abdominal infection, can be missed if the clinical assessment stops with a urinalysis and a positive urine culture.

Table 37.2 Host defenses and external selective pressures influencing the outcome of interactions between bacteria and the urinary tract

Parameter	Nonspecific	Genitourinary specific
Host defense	- Intact skin - White blood cell function (e.g., phagocytosis) - Age - Neoplasia - Immune competence - Hormonal changes - Pregnancy - Malnutrition - Malaria, diabetes - Psychological state - Immunodeficiency syndrome	- Diabetes mellitus - Pregnancy - Sickle cell anemia (black women) - Neuromuscular diseases - Structural abnormality - Gout (?) - Hypertension (?) - Potassium deficiency (?) - Urine pH - Urine flow amount - Urinary tract mucosa
Selective pressures	- Birth control pills - Smoking - Alcoholism - Anesthesia - Drug addiction - Immunosuppressive drugs - Irradiation, therapeutic - Antimicrobial therapy	- Sexual activity - Incontinence - Bladder catheterization, indwelling or intermittent - Instrumentation - Urinary stones - Urine retention

Pregnancy

Pregnant women are at higher risk for UTI for several reasons. Hormonal changes lead to changes in the ureter and urethra, making them more susceptible to bacterial adherence and infection. In addition, the enlarging uterus can put pressure on the bladder and impair urinary flow, leading to cystitis or pyelonephritis. ASB also may occur, but in pregnant women this should be treated because infection can lead to premature labor as well as infectious complications in the fetus or the newborn. Susceptibility testing is particularly important in this patient population because not all antimicrobials can be given to pregnant women. This is particularly true of drugs such as quinolones, which are a standard treatment for UTIs in the general population. Pregnant women should be screened closely for signs of a UTI and, at the third trimester, be screened again for organisms that can cause UTIs and be transmitted during vaginal delivery of her child. Early identification and treatment of UTI in pregnant women has decreased the incidence of preterm birth and low-birth-weight neonates.

Renal transplantation

For decades, the number of kidney transplantations in the United States has continued to increase, with nearly 25,000 transplants occurring in 2021. Although kidney transplantation can be lifesaving and improve quality of life, significant complications can occur, including infection of the urinary tract. UTI is the most common infection in kidney transplant recipients, occurring in up to 86% of recipients. Several factors contribute to this increased risk of UTI, including significant immunosuppression; foreign bodies, such as stents and urinary catheters; stricture; obstruction; scarring or other abnormalities of the ureter and urethra; graft trauma; and diabetes mellitus. In fact, having a ureteral stent can increase the risk of UTI by 1.5-fold in this population. In addition, because these patients are receiving trimethoprim-sulfamethoxazole prophylaxis for *Pneumocystis* pneumonia as well as for UTIs, they can develop infection with organisms resistant to this antimicrobial.

UTIs in kidney transplant recipients most often present as cystitis; however, almost 25% of cases may present as pyelonephritis and can lead to allograft injury. In addition, UTIs may be responsible for more than 50% of bacteremias in this population. These patients might have atypical presentations, lower colony counts, and variable pyuria. *Escherichia coli* and other gram-negative bacilli account for up to 90% of the pathogens in culture positive UTIs in kidney transplant recipients. However, some transplantation centers are now reporting a rise in enterococcal UTIs, particularly in the first month after transplantation.

Bladder catheterization

UTIs are the most common healthcare-associated infections, accounting for approximately one third of these infections. Up to 95% are related to bladder catheterization and/or manipulation. In a catheterized patient, the risk of acquiring a UTI depends on the duration of catheterization, appropriate catheter care, and host susceptibility. Bacteriuria will inevitably occur, given enough time, because the mechanisms of

infection and colonization involve intraluminal and extraluminal migration of bacteria. The most common pathogens include enteric gram-negative bacteria (particularly *E. coli*), enterococci, yeasts, and staphylococci. Candiduria is a common finding in patients with long-term catheterization, and its clinical relevance is difficult to determine and controversial, particularly in asymptomatic patients.

Hospitals and long-term care facilities implement protocols to care for and monitor patients with catheters to ensure that UTIs are not associated with prolonged use of a bladder catheter. Infections associated with catheters (i.e., CAUTIs) are reportable to accreditation and government agencies. When facilities have a higher-than-normal CAUTI rate, this affects the overall “score of care” and the insurance reimbursement rates it receives for patient care. Certain professional organizations, such as the Infectious Diseases Society of America (IDSA), have published guidelines to reduce CAUTI occurrence and manage patients with CAUTIs and catheter-associated ASB infections.

Sexually transmitted infections

Although most sexually transmitted infections (STIs) involve the genital tract, the anatomic proximity to the vagina in females and the shared tract in males lends itself to UTIs caused by the same organisms implicated in an STI. Often, the symptoms of STIs and UTIs are shared, and the infected person may present with complaints of either a urinary tract or genital tract infection. Most organisms that cause STIs are fastidious; therefore routine urine culture methods may not recover these organisms. In a study of female patients presenting to an urban emergency department, STI-associated organisms were found in 17% of urine cultures collected by catheterization; of these, they had colony counts that would exclude reporting them based on widely shared microbiology laboratory guidelines. Some organisms, such as *Mycobacterium*, *Chlamydia*, and *Ureaplasma* species, can cause UTIs secondary to STIs but cannot be cultured or would be difficult to grow, making sensitivity of detection on urine cultures extremely poor. A UTI secondary to an STI should be considered if the patient is sexually active, has multiple partners, has a past medical history of STIs with unresolved clinical symptoms after empiric therapy, and has negative urine cultures.

Clinical signs and symptoms

UTIs in children younger than 2 years of age usually manifest with nonspecific symptoms, such as failure to thrive, vomiting, lethargy, and fever. Children older than 2 years of age are more likely to complain of more localized symptoms, such as dysuria, frequency, and abdominal pain.

Adults with uncomplicated lower UTIs limited to the urethra or bladder present primarily with dysuria, often in combination with frequency, urgency, suprapubic pain, and hematuria. Each episode of uncomplicated UTI in women is usually associated with 1 week of symptoms. An acute, complicated UTI presents with the same symptoms seen in uncomplicated UTIs with the exception of lower back and/or flank pain associated with pyelonephritis may be reported. Patients with U-UTIs, such as pyelonephritis, may also present with nausea, vomiting, fever, chills, night sweats, and

costovertebral angle tenderness. These symptoms may occur in the presence or absence of symptoms of cystitis. Dysuria and frequency may precede the onset of upper urinary tract and systemic symptoms by 1 or 2 days.

Bacteremia, when present, may help confirm a diagnosis of pyelonephritis. However, presenting symptoms of UTI can differ widely. Cases of pyelonephritis may be asymptomatic, manifest symptoms such as those of lower UTIs, or present as life-threatening sepsis. Most older adult patients have atypical presentations, such as delirium, failure to thrive, and/or weakness. Transplant recipients also may present atypically and might have pain at the allograft site.

Although dysuria is the most common reason for obtaining a urine specimen for culture, this clinical presentation is neither sensitive nor specific. Dysuria may be present in infections with herpes simplex virus, BK virus in kidney transplant patients, adenovirus, cytomegalovirus, *Ureaplasma*

urealyticum, *Chlamydia trachomatis*, or *Neisseria gonorrhoeae*. These agents are not detected by routine bacteriologic urine culture. Many noninfectious conditions, including urethral inflammation from physical or chemical agents or because of trauma, may have similar symptoms. Flank pain and fever without lower urinary tract symptoms, and bacteremia without urinary tract symptoms can be seen in patients with indwelling urinary catheters. Fig. 37.3 depicts an evaluation scheme for women with acute dysuria.

Causes of urinary tract infections

Pathogenesis

Bacteria can gain access to the urinary tract by the ascending route or the hematogenous (or descending) route. Women

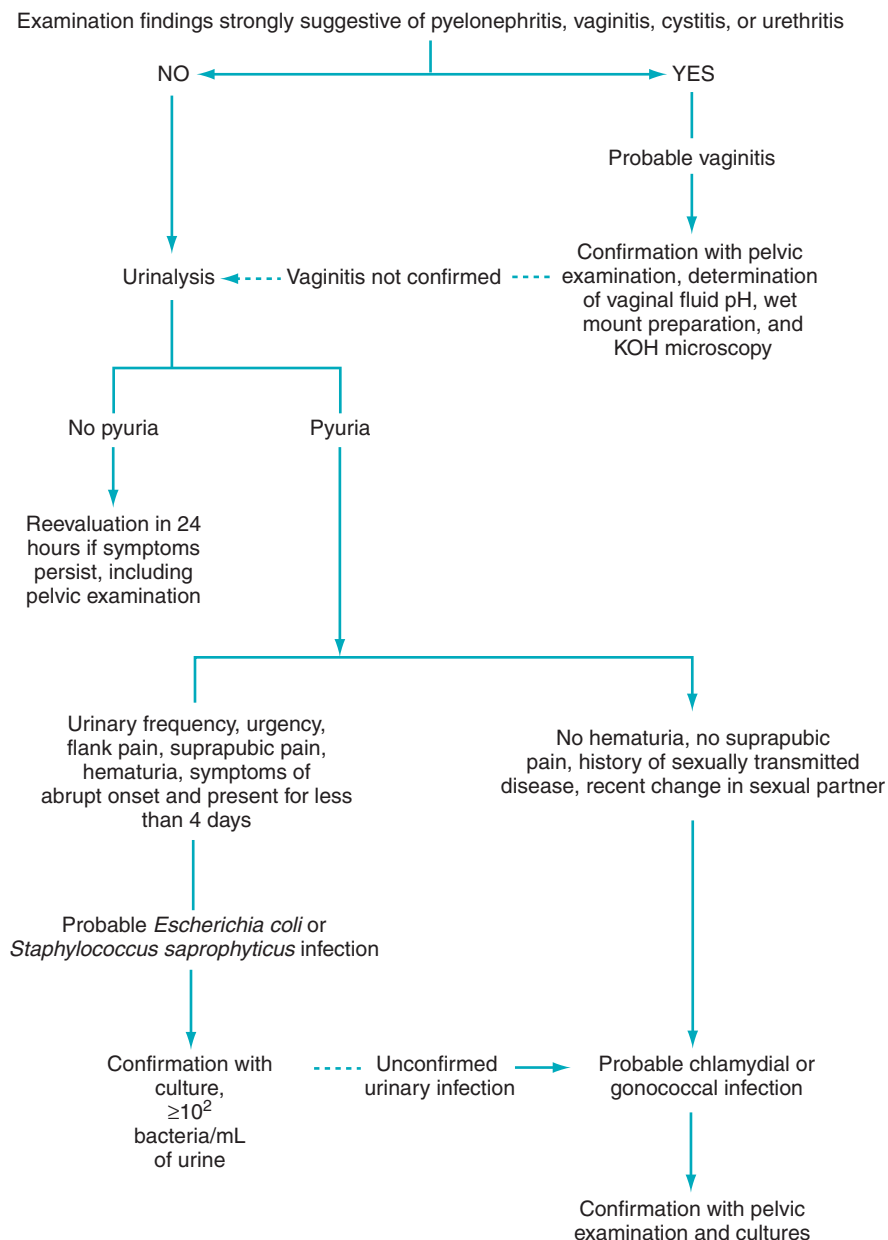


Fig. 37.3 Evaluation of women with acute dysuria. KOH, Potassium hydroxide.

usually acquire UTIs via the ascending route. Because of a shorter ureter in women, bacteria are easily introduced into the bladder through sexual intercourse or proximity of the urethral opening to the anus. Once established in the bladder, bacteria ascend the ureters, probably aided in many cases by the inflammation of the GU tract musculature. Some of the most common etiologic agents in UTIs also are motile because of flagella, which is theorized to aid in movement upward into the urinary tract.

U-UTIs caused by many species of gram-positive bacteria, mycobacteria, and fungi occur via hematogenous spread. Isolation of these organisms should alert a health care provider to consider sepsis and determine the primary source of the infection, as the UTI is a secondary manifestation (refer to the Case in Point). Gram-negative infections via the hematogenous route are more difficult to assess. Of these possible routes of infection, the ascending route is of paramount importance and most often associated with gram-negative bacteria. The hematogenous route is a less frequent but significant pathway with long-reaching implications including bacteremia and heart valve infections.

Causative agents

The normal bacterial biota of the skin, which includes the area surrounding the urethra, consists mostly of *Staphylococcus epidermidis*. Table 37.3 presents the organisms that do not grow well in urine and are not common causes of UTIs, arranged in descending order by age or patient status. Those listed first are found most commonly, and those at the end are less frequently isolated. Box 37.3 lists the more readily recognized agents of UTIs and emerging uropathogens that must be

recognized given the dynamics of urinary tract pathogens, population at risk, and selected pressures, as noted earlier. These organisms present unique challenges to clinical microbiologists.

Gram-negative bacilli

Of the Enterobacterales, antimicrobial-susceptible strains of *E. coli* that exist in the patient's fecal biota cause the majority of uncomplicated UTIs. As antimicrobial-resistant members of the Enterobacterales become more common, UTIs with multidrug-resistant bacteria are also increasing. Hospitalization and catheterization duration increases the risk of developing an infection with other organisms, such as *Pseudomonas aeruginosa*, *Acinetobacter* spp., *Proteus* spp., *Klebsiella oxytoca*, and *Enterobacter* spp. Infections can be polymicrobial, especially in complicated UTIs, and can yield urine cultures that are positive for multidrug-resistant organisms. Though not as common, group B *Streptococcus* (GBS) in pregnancy can pose a problem. Vaginal colonization of GBS is associated with neonatal sepsis with meningitis, preterm rupture of membranes, and early labor. Screening for GBS is part of routine prenatal health care.

Gram-positive cocci

Among the gram-positive cocci, *Enterococcus faecalis*, *Enterococcus faecium*, and *Staphylococcus saprophyticus* are the most commonly encountered causative agents. Enterococcal UTIs occur primarily in older men, particularly in association with urinary tract manipulation or instrumentation or prostatic hypertrophy. *S. saprophyticus* is found predominantly in

Table 37.3 Microbiota of normal voided urine^a defined by patient's age and status

Patient age, status	Usual biota
Neonate	Sterile
1–3 days of age	Staphylococci Enterococci Diphtheroids <i>Mycobacterium smegmatis</i>
Prepubertal	Micrococci Streptococci (α-hemolytic and nonhemolytic) Coliforms Diphtheroids
Adult	<i>Lactobacillus acidophilus</i> <i>Staphylococcus epidermidis</i> Streptococci (α-hemolytic and nonhemolytic) <i>Escherichia coli</i> Diphtheroids Yeasts Anaerobic streptococci <i>Listeria</i> spp.
Pregnancy	<i>Lactobacillus acidophilus</i> <i>S. epidermidis</i> Yeasts
Postmenopausal	Resembles prepubertal biota

^aUsually sterile or fewer than 1000 colonies/mL.

BOX 37.3 Recognized microbial agents of urinary tract infections

Common agents

Enterobacterales (especially *Escherichia coli*)
Enterococci (including vancomycin-resistant enterococci)
Streptococcus agalactiae (group B *Streptococcus*)
Pseudomonas spp.
Staphylococcus aureus
Staphylococcus saprophyticus
Candida spp.

Less common agents

Gardnerella vaginalis
Ureaplasma urealyticum
Mycoplasma hominis
Mobiluncus spp.
Leptospira spp.
Mycobacterium spp.
Chlamydia trachomatis (in males)

Agents often associated with multisystem diseases

Salmonella (with gastroenteritis)
Schistosoma haematobium
Cryptococcus neoformans
Trichosporon beigeli
Trichomonas vaginalis
Aspergillus spp.
Penicillium spp.
Adenovirus
Herpes simplex virus

Table 37.4 Urinary tract infections caused by coagulase-negative staphylococci

Characteristics of infections	Organism	
	<i>S. epidermidis</i>	<i>S. saprophyticus</i>
Sex of affected patient	Men and women equally	Women 95%, 16–35 years of age
Population at risk	Hospitalized patients with urinary tract complications	Healthy outpatients
Incidence	Common—20% or more of all UTIs for hospitalized patients >50 years of age	Uncommon—3.5% or fewer of all UTIs in hospitalized patients
Presentation	90% asymptomatic	90% symptomatic; indistinguishable from <i>Escherichia coli</i> UTIs
Therapy	Often resistant to multiple drugs	Responds readily to traditional urinary tract antimicrobials except nalidixic acid
Outcome	Bacteriuria often persists after therapy	Relapse rare; occasional reinfection

UTI, Urinary tract infection.

symptomatic sexually active women younger than 40 years of age. *S. epidermidis* is found in hospitalized patients older than 50 years of age. These individuals usually have had recent urinary tract surgery, indwelling urinary catheters, or chronic urinary tract disease. *S. epidermidis* is associated with UTIs in only about 20% of cases, and it is debatable if it is truly the causative agent. Table 37.4 highlights important clinical features of *S. epidermidis* versus *S. saprophyticus* presentation and demographics in UTIs.

S. aureus is also an important uropathogen. This bacterium is usually directly correlated with seeding from another system, such as blood (hematogenous route), and warrants additional investigation such as blood cultures and imaging studies to locate the primary source of infection. Because methicillin-resistant *S. aureus* is common, the relevance of isolation in urine cultures is acutely important in management of patient treatment and care.

Gram-positive bacilli

In rare cases, *Listeria monocytogenes* can be isolated from the urine of infants who acquired a perinatal infection with this organism and in 1% of patients with a systemic infection or renal transplant. *L. monocytogenes*, diphtheroids, and mycobacteria cause diseases predominantly in selected patient populations and often in association with a bacteremia. If any of these organisms are recovered from urine, consultation with the health care provider helps assess the significance because contamination is more likely. In select situations, blood cultures or cultures from other sites may help determine the significance of the isolate. Although rarely associated with UTIs, mycobacteria have added significance in patients with human immunodeficiency virus (HIV) infection or those who are otherwise immunosuppressed.

Fungi

Candiduria is rare in healthy adults, with one 10-year retrospective study revealing up to a 0.75% rate; however, candiduria is more commonly seen in hospitalized patients. Up to 10% of patients in tertiary care facilities have *Candida* in their urine. Over the past few decades, healthcare-associated candidal UTIs have increased, with fluconazole resistance, a

drug commonly used to treat *Candida*, also increasing. *Candida albicans* is the most clinically significant species. Of particular concern is the globally emerging *Candida auris*. This yeast is highly drug resistant and virulent with a mortality rate up to 60% in a variety of infections. *Candida dubliniensis* is a rare isolate most often seen in patients who are immunocompromised. Predisposing factors for *Candida* UTI include diabetes mellitus, antimicrobial and corticosteroid therapy, incidence of urinary instrumentation, female sex, and disturbance of urine flow. Most candidal UTIs are acquired via the ascending route.

Other yeasts that can be isolated in urine cultures include *Cryptococcus* spp. and dimorphic fungi. These are very rare occurrences and generally present in a manner that would involve other types of cultures, such as CSF or tissue cultures, or other diagnostics tests to supplement the finding. The isolation of other pathogenic fungi, such as *Blastomyces dermatitidis*, *Coccidioides immitis*, and *Histoplasma capsulatum*, in urine is highly significant and indicative of disseminated infection regardless of colony count.

Most yeasts are evident in culture within 2 to 5 days; however, urine cultures are generally held for no more than 3 days. If the health care provider suspects fungal involvement, it is better to order a fungal culture that may be held for up to 8 weeks rather than a standard bacterial urine culture. Consultation between laboratory scientists and the health care provider are best in these cases.

On agar media, early colonies of *C. albicans* can resemble colonies of coagulase-negative staphylococci, requiring a review of Gram-stained smears to avoid misidentification. Because *Candida* spp. often are recovered from hospitalized patients with indwelling catheters, incorrect identification as bacteria, rather than a yeast, results in a susceptibility report indicating broad antimicrobial resistance and can grossly affect patient care. A wet mount preparation examined under $\times 10$ magnification also can provide a rapid identification. Older colonies, 48 hours or more, may exhibit “feet” or “starbursts” around the colonies, which is indicative of *Candida* spp. A germ tube assay for the presence or absence of pseudo-hyphal elements can help speciate the *Candida* spp. into *C. albicans/dubliniensis* or others. *C. auris* is thought to be misidentified by traditional methods. Matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry is likely the most accurate identification method.

Although findings of candiduria may not require antifungal therapy, particularly in catheterized patients, properly collected urine specimens yielding *Candida* spp. should be considered abnormal. Candiduria may be an indication of bladder or renal parenchymal infection, urinary tract fungus ball, or disseminated candidiasis.

Optimal management of candiduria is still debated. A paucity of data regarding the efficacy of antifungal agents in the urinary system exists. One study involving hospitalized patients with candiduria (asymptomatic or minimally symptomatic) showed that the antifungal agent fluconazole was effective for short-term eradication, but long-term eradication rates were disappointing. Experience has shown that removal of catheters is effective in eliminating candiduria in catheterized patients.

Case check 37.3

Common causative agents of UTIs include coagulase-negative staphylococci, *E. coli*, *Klebsiella*, other Enterobacterales, and enterococci. *Pseudomonas* spp. and *Candida* spp. are also seen, especially in healthcare- or catheter-associated UTIs.

Other agents of urinary tract infections

Anaerobes, common microbiota in the urethra, are not usually responsible for UTIs but may be significant when isolated from suprapubic aspirates. *C. trachomatis* and *N. gonorrhoeae* can cause urethritis, cystitis, and prostatitis. This is particularly an issue with women, in whom it is clinically difficult to distinguish between urethritis and **cervicitis**. Recently, *Mycoplasma* and *Ureaplasma* organisms have been associated with UTIs, particularly in neonates from lower socioeconomic groups and in individuals with HIV infection.

With recent advances in total laboratory automation (TLA), the speed at which urine cultures are inoculated to media is faster than in traditional manual setups. This has enabled microbiology laboratories to recover urinary pathogens that otherwise may have perished before inoculation was performed. From this and the combination of more accurate identifications attributed to MALDI-TOF technology, more isolates of *Alloscardivia omnicolens*, *Actinomyces* spp., and *Actinotignum schaalii* (formerly *Actinobaculum schaalii*) have been isolated with enough consistency to warrant these as emerging or novel pathogens.

Identification of *Gardnerella vaginalis* in urine commonly reflects vaginal contamination. However, this organism is also gaining credibility as an emerging urinary tract pathogen as repeated reports emerge in which this microbe is the primary isolate. *G. vaginalis* is typically cultured in older adult women, suggesting it may be associated with a postmenopausal change. Certain organisms, particularly *Salmonella* spp., are involved with multiple organ systems; these may be part of a primary disease or can arise secondarily. To a lesser extent, species of *Enterobacter*, *Morganella*, *Serratia*, *Hafnia*, *Kluyvera*, *Acinetobacter*, *Lactobacillus*, and *Streptococcus* have all been implicated in UTI.

Viral agents, especially adenovirus and herpes simplex virus, have also been associated with cystitis and should be sought when the clinical situation warrants. Although

Table 37.5 Most common causative agents of urinary tract infections associated with frequent clinical presentation (disease syndromes)

Clinical presentation	Common causative agents
Upper urinary tract infections	
Acute pyelonephritis	Enterobacterales <i>Staphylococcus aureus</i>
Subclinical pyelonephritis	Coagulase-negative staphylococci <i>Candida</i> spp. <i>Mycobacterium</i> spp. <i>Mycoplasma hominis</i>
Lower urinary tract infections	
Acute bacterial cystitis	<i>Escherichia coli</i> <i>Klebsiella</i> spp. Other members of the Enterobacterales Enterococci Coagulase-negative staphylococci
Urethritis	
Acute urethral syndrome	<i>Chlamydia trachomatis</i> <i>Neisseria gonorrhoeae</i> <i>Mycoplasma genitalium</i> <i>Ureaplasma urealyticum</i>
Other infections	
Gonococcal urethritis	<i>N. gonorrhoeae</i>
Chlamydial urethritis	<i>C. trachomatis</i>
Vaginitis	<i>Gardnerella vaginalis</i> and <i>Mobiluncus</i>
Prostatitis	Resembles those of acute bacterial cystitis
Catheter-associated (hospital-associated) urinary tract infection	<i>E. coli</i> <i>Klebsiella</i> spp. <i>Proteus mirabilis</i> <i>Pseudomonas</i> spp. <i>Candida</i> spp.
Chronic or recurrent (inpatient/outpatient) urinary tract infection	<i>E. coli</i>

exceedingly rare outside central Africa, *Schistosoma haematobium* is the only parasite known to be detected in urine. The eggs tend to lodge in the urinary tract causing chronic infections and increased risk of bladder cancer. Table 37.5 presents organisms associated with UTIs, listing the uropathogens usually associated with a particular clinical presentation or disease syndrome.

Laboratory diagnosis

Significance of colony counts: historic background

The urinary tract above the urethra in normal healthy individuals is sterile, as is urine, unlike the urethra, which is colonized with perineal microbiota. In women, vaginal biota can contaminate the urine, but many of the organisms that colonize the vagina are also implicated in UTIs. Therefore it

could be difficult to know whether growth in urine cultures is indicative of infection or contamination.

For decades, quantitative urine culture determination has been considered one of the more straightforward and simple laboratory tests to diagnose UTIs. It was accepted that a finding of 10^5 CFU/mL or more symbolized infection. In his 1956 classic study, Kass showed a clear distinction between the number of bacteria in the urine of asymptomatic or symptomatic women with pyelonephritis and those who were uninfected. When infected, 95% had colony counts higher than 10^5 CFU/mL. All specimens were collected by catheterization (not voided), most asymptomatic women had counts below 10^3 CFU/mL, and the prevalence of infection was only 6%. With this low prevalence rate, the number of false-positive results would be high and hence the 10^5 CFU/mL positive infection cutoff point. The sensitivity was only 51% for women with clinical pyelonephritis who had colony counts above 10^5 CFU/mL.

In the 1960s and 1970s, additional studies began to challenge Kass's original work. Several investigators showed that among symptomatic women with acute lower UTIs (urethra, bladder, or both), 29% to 45% had colony counts lower than 10^5 CFU/mL by suprapubic aspiration. These women had **dysuria** and **pyuria**, yet unexpectedly negative cultures by the traditional Kass-based criterion (e.g., more than 10^5 CFU/mL signifying infection). In 1980, **acute urethral syndrome**, or urethritis, was more clearly defined as one of three causes of acute dysuria with pyuria. The other causes were vaginitis and cystitis. The causative organisms included classic coliforms (rapidly growing, lactose-fermenting, gram-negative bacilli), such as *E. coli*, and *S. saprophyticus* at colony counts above 10^3 but below 10^5 CFU/mL. Simultaneously implicated in sexually active women were the emerging nongonococcal urethritis pathogens *C. trachomatis* and *U. urealyticum*. Thus in women with UTIs, as many as 50% had urethral syndrome—not cystitis—and had negative culture results using traditional laboratory methods.

In 1982, Stamm et al. reexamined the diagnostic criteria for women with acute symptomatic lower UTIs. In contrast with Kass's classic work, Stamm's study included coliforms at a threshold above 10^2 CFU/mL, which provided a sensitivity of 95% and a specificity of 85%. The presence of pyuria in uncentrifuged urine specimens was a sensitive adjunct. The prevalence of coliform infections was 36% in women evaluated by Stamm compared with Kass's 6%. Because of the higher infection prevalence, the positive predictive value (PPV) of infection also increases, and the number of false-positive results decreases. Stamm used a low cutoff of 10^2 CFU/mL to differentiate infection from contamination.

In the late 1980s, because of the emerging significance of pyuria, investigators reevaluated the accuracy of the classic urinary sediment microscopic examination often performed as part of a urinalysis. There was concurrence that microscopic urine screening for detection of UTI was inherently inaccurate, not reproducible, and had no direct correlation with clinically proven UTIs, so microscopic examination of urine specimens should be reserved for the detection of casts and crystals. Furthermore, urinalysis assessment of WBCs could not be correlated with the actual leukocyte excretion rate (WBCs per microliter) measured by hemocytometer chamber count. When clinical studies using the latter method

of determining pyuria were reviewed, the following conclusions emerged:

- A urine leukocyte count of $10/\mu\text{L}$ or higher occurs in less than 1% of asymptomatic patients without bacteriuria but in more than 96% of symptomatic men and women with significant bacteriuria.
- Most symptomatic women with pyuria but without significant bacteriuria have UTIs with bacterial uropathogens present in colony counts from 10^2 to 10^5 CFU/mL or have infections with *C. trachomatis* or *U. urealyticum*.
- Women with ASB with pyuria probably should be categorized as having true infection, whereas women with ASB with no pyuria are categorized as having transient self-limited bladder colonization.
- Most patients with catheter-associated bacteriuria also have pyuria—hence infection.
- Simultaneously, an impregnated paper strip that measured urine leukocyte esterase was introduced and found to correlate well with hemocytometer chamber counts. The leukocyte esterase test is inexpensive and quick (1 minute) and requires no technical skills or equipment.

The significance of these conclusions was not readily apparent. Until recently, the colony count was regarded as the gold standard for determining whether a patient had a real and treatable UTI. However, the urine bacterial colony count cannot stand alone as a single criterion when evaluating a patient for the presence or absence of UTI. Urine culture is requested not only in connection with acute UTI symptoms but also in the absence of specific symptoms; as a test of cure; to detect ASB in pregnant women; and to evaluate patients for bacteremia, fever, or both.

The criteria that determine whether a UTI is present must include the presence or absence of symptoms, predisposing factors, patient population, and type of organism(s) isolated. Therefore the outcome of a urine culture must be evaluated along with other laboratory and clinical data. Attempts to attach significance to the colony count should be restricted to the original patient population in which that significance was established, that is, asymptomatic individuals with pyelonephritis.

Specimen collection and transport

Preventing contamination by normal vaginal, perianal, and interior urethral biota is the most important consideration when collecting a clinically relevant urine specimen. The microbiology laboratory will determine the significance of an isolate based on the total number and types of colonies present. The colony count is also useful for the health care provider who may want to determine the primary organism present and the role of treatment. Therefore all necessary precautions should be taken to ensure that the colony count represents the number of organisms present in urine when the specimen was collected and not contaminants.

Urine samples should arrive in the laboratory at room temperature, and culture plates should be inoculated within 2 hours of collection unless boric acid has been added to the urine as a preservative. Specimens can be stored refrigerated for up to 24 hours. When a specimen arrives at the laboratory, it must be labeled as to location or timing, and this information must accompany the results for proper interpretation. It

is incumbent on laboratory directors to define specific criteria for collection and transport and, within their realm of responsibility, ensure that these protocols are followed. Culture media and pathogen reporting can vary depending on the source of the urine.

Voided midstream specimen collection

A voided midstream collection, also known as a *clean catch*, is the most commonly used method in clinical practice. The urine is contaminated with bacteria from the urethra unless the first portion of the voided specimen is discarded, hence midstream. Patients who self-collect clean-catch midstream urines must be instructed to use an antimicrobial wipe before collection and, for women, to spread the outer labia to ensure that the urine stream does not include skin biota. Voided urine collection kits should contain instructions for the patient on proper specimen procurement, and instructions should be provided both orally and in print. Patient education should be part of the specimen processing because proper collection has a considerable influence on the result of subsequent laboratory procedures. It has also been reported that diagrams showing the way a specimen should be collected are much more easily understood by the patient compared with written instructions.

Catheterized specimen collection

Catheterized specimen collection, an invasive technique, reduces the risk of contamination of urine by the urethral biota; however, because the catheter passes through the urethra, some contamination can occur. Before urine is collected with a single straight catheter, the urethral opening or vaginal vault is cleansed with a soap solution and rinsed with sterile water. The initial urine flow is discarded because it may contain organisms acquired as the catheter passed through the urethra.

Specimens may also be collected from an existing indwelling urinary catheter by cleaning the catheter collection port with an alcohol pad and puncturing it directly with a needle and syringe. The specimen should never be collected from the Foley drainage bag or output line itself. The longer the patient has the indwelling catheter in place, the more likely the urine sample may be contaminated from biofilms that grow on the walls of the catheter. For this reason, urine cultures from indwelling catheters are best obtained after the catheter is inserted and no more than 3 days postinsertion.

Samples obtained from an ileoconduit are collected from the stomal opening after the area has been swabbed with an alcohol wipe. The urine on the external appliance is never used for culture because, like urine in the drainage bag in patients with indwelling catheters, it is often contaminated. The bacteriologic results of urine specimens collected by cystoscopy (bilateral urethral catheterization) or by bladder washout are used to localize the infection to the upper or lower urinary tract and, in the former case, to the left or right kidney. Specimens obtained by straight catheterization, bilateral urethral catheterization, or bladder washout or from an ileoconduit may be submitted in a tube or broth unless an assessment of pyuria is necessary.

Suprapubic aspiration

Suprapubic aspiration is the definitive method for collecting uncontaminated specimens. Generally, any organism isolated

from these specimens is considered clinically significant; however, this may not be correct because transient colonization of the bladder can occur. Suprapubic aspirations are collected primarily from infants and patients in whom the interpretation of the results of voided specimens is difficult for various reasons.

Alternatively, in infants and young pediatric patients, urine collections are occasionally submitted in a pediatric urine collection bag in which a sterile bag with an opening is adhered to the cleansed genital region of the child. Urine collected via this manner is acceptable for urinalysis, but the utility for microbial culture remains uncertain. Should the laboratory director decide to accept pediatric urine bags as a source, they should consider placing a comment on the final report as the contamination rate in bagged specimens is >50% compared with when a straight catheter sample is obtained. Suprapubic aspiration of urine specimens is the only suitable method to obtain an anaerobic culture. Following skin antisepsis, urine is collected from a full bladder by using a needle and syringe.

Other considerations

In addition to the way the specimen is collected, other parameters can have an impact on the suitability of the specimen and interpretation of culture results.

Urine volume

The volume of urine received is rarely a problem for routine bacteriologic culture. Detection of significant pyuria by sediment examination requires at least 10mL of specimen. Alternative methods of pyuria detection may be needed when smaller volumes are received. At least 20mL of urine may be required to recover mycobacteria or fungi. If these agents are strongly suspected and previous specimens of lesser volume have tested negative, a single, early-morning collection of at least 20 to 40mL should be obtained for culture. However, 24-hour collections for urine specimens in microbiology are unacceptable.

Number of specimens and timing of collection

The number of specimens and the timing of urine collection depend on the patient's clinical state and collection method used. A single, clean-catch, voided specimen or single specimen obtained by catheterization in a symptomatic patient is usually sufficient in the setting of pyuria and a culture revealing a uropathogen. In symptomatic patients, antimicrobial therapy is often instituted immediately after urine collection and is not withheld while waiting to procure subsequent specimens. For purposes of optimal yield, particularly for mycobacteria, it has been recommended that only first-morning specimens be processed; if such specimens are not available, the urine should be allowed to incubate in the bladder for as long as possible (at least 4 hours) before collection to increase the bacterial density and therefore culture sensitivity. *S. haematobium* parasite eggs are shed based on the circadian rhythm of their human host and are present at the greatest concentration at noon. Therefore urine for detection of *S. haematobium* is best collected around midday and adjusted if the patient has recently returned from another global time zone.

In the absence of symptoms, a single, first-morning voided urine specimen from a pregnant woman is sufficient to detect ASB. In this case, one or two more first-morning specimens may be collected on separate days to demonstrate the significance of the isolates. Because quantitation is necessary for

the diagnosis of ASB and because an asymptomatic condition does not require immediate therapeutic intervention, these specimens should be limited to first-morning collections. Urine specimens from other asymptomatic patient populations, except those demonstrating significant pyuria, cannot be reliably interpreted.

Additives

Additives to urine specimens are designed primarily to preserve the bacterial density present at collection. These additives maintain the original colony count during ambient temperature transport until quantitative cultures can be accomplished, obviating the need for refrigerated transportation, which can be destructive to certain fastidious pathogens such as *N. gonorrhoeae*.

Sodium borate (boric acid) maintains the sample integrity for up to 48 hours after collection at room temperature, preventing overgrowth of nonpathogenic biota without impairing growth of existing pathogens. Liquid preservatives have been associated with dilution errors and decreased recovery of organisms at 24 and 48 hours. Lyophilized preservation is less inhibitory but requires a urine volume of at least 3 mL to avoid pathogen inhibition. However, lyophilization requires instrumentation that is not routinely available to most provider offices and hospitals.

The effects of preservatives on the detection of pyuria have not been adequately determined. Regardless of the preservative used, the maximum time from collection to processing should not exceed 24 hours. In addition, the effect of preservatives on selected manual and automated TLA methods (see later) has not been extensively evaluated. The importance of identifying pyuria in many cases and the lack of evidence that urine preservatives allow an accurate leukocyte count after 2 hours argue against the use of preservatives for specimens from symptomatic patients.

Microbial detection

Specimen screening: rapid nonculture methods

Often, urinalysis of clean-catch specimens is a multistep tiered algorithm that begins with a urinalysis dipstick for the presence of nitrites, blood, and/or leukocyte esterase, and urine pH. Leukocyte esterase and nitrite tests have low sensitivity, especially when low numbers of bacteria are present. If there are indications of infection from the dipstick results, a microscopic examination of the urine is performed. In a microscopic examination, the presence of bacteria, yeast, erythrocytes, and leukocytes, as well as casts and urine crystals can determine if further investigation is necessary. This method can also reveal the presence or absence of bacteria or fungi. The presence of struvite crystals, caused by the formation of magnesium ammonium phosphate crystals at basic urine pH, may be indicative of infections caused by *Proteus* or *Klebsiella* species, which produce urease and alter the normal pH of urine.

Dipstick urinalysis and microscopic examination should be reported within 2 hours after collection of a urine specimen. Based on the results of the microscopic examination, microbial cultures could be indicated. Although this is a

common algorithm, the specific parameters causing reflex to the next diagnostic assay varies among laboratories. Should the patient's situation dictate a deviation from the algorithm, many laboratories also give the health care provider discretion to independently order each assay. Because timing is important for interpretation of the preliminary and final reports, the collection time, time received by the laboratory, and time to final report should be disclosed. In addition to providing important information to the health care provider early in the diagnostic analysis, a tiered algorithm has shown to decrease the frequency of negative urine cultures, decrease workload in the microbiology laboratory, and improve antimicrobial stewardship by de-escalating antimicrobial use.

Many providers will initiate treatment based on the knowledge of which organisms and their antimicrobial susceptibility patterns (cumulative antibiograms) are circulating in the local community. Each laboratory strives to publish an antibiogram, which is a summary of the number, sources, and antimicrobial profiles of pathogen isolates each year. As preliminary reports are updated, the treatment can be further refined.

Several key points should be considered when using screening methods:

- Screening methods capable of detecting bacterial densities of 10^5 CFU/mL or higher are appropriate for the detection of ASB in pregnant women.
- False-positive results occur more often with methods that test for multiple parameters (e.g., bacteria and WBC count).
- Methods detecting significant pyuria and bacteriuria with sensitivities of 50 to 100 leukocytes/ μ L and 10^2 CFU/mL may be appropriate for screening voided urine specimens or those from indwelling urinary catheters in symptomatic patients.
- Screening methods for urine collected by invasive means should be considered for microbiology culture whether or not a tiered algorithm is used. This includes straight catheterization, cystoscopy, suprapubic aspiration, or bladder washout specimens.

Manual urine screening methods

Detection of bacteria when pyelonephritis is suspected

Microscopic examination of Gram-stained smears of urine samples is a debatable practice because of low sensitivity. Also, if staining is performed, it is not recommended to report the microscopic morphology of the organisms because of the low specificity and the inability to distinguish viable from nonviable organism and pathogens from contaminants. Gram-stained smears can reveal the presence and absence of bacteria or yeast, but they generally cannot distinguish between monomicrobial or polymicrobial infections, especially if the morphology of two or more pathogens is the same.

When microscopic analysis is performed, urine concentration and sediment analysis or cytospin technology is best. The presence of one or more bacterial cells per oil immersion field in at least five fields in a smear of uncentrifuged urine correlates with more than 10^5 CFU/mL. If the uncentrifuged preparation is negative, the sediment preparation for leukocyte examination should be stained. Bacterial cells seen in this preparation indicate a density of fewer than 10^5 organisms/mL and, in the

Table 37.6 Manual screening methods, principles of assay, and threshold of detection for urinary tract infections

Screens	Principle	Reported threshold of detection (CFU/mL)
Manual		
Microscopy Direct, uncentrifuged or centrifuged (cytospin)	Recognition of organism morphotypes and Gram stain	1-2 organism/OIF = 10^5
Chemical		
Enzymatic dipstick		
Nitrate reductase (Griess test)	Gram-negative bacteria reduce nitrates to nitrites	
WBC leukocyte esterase	Measures presence of WBC enzyme	Equivalent to 5 WBC/hpf
Chemstrip LN	Combination testing of nitrate and esterase assays	$>10^4$ – 10^5
Enzyme tube		
Uriscreen ^a	Measures catalase present in bacteria and somatic cells	$<10^4$ – 10^5
CFU, Colony-forming unit; hpf, high-power field; OIF, oil immersion field; UTI, urinary tract infection; WBC, white blood cell.		
^a Accutest Uriscreen, Jant Pharmacal Corporation, Encino, CA.		

presence of clinical findings of acute pyelonephritis, may suggest urinary obstruction or perinephric abscess. The presence of bacteria or fungi assists in the selection of an appropriate antimicrobial therapy. Table 37.6 lists various manual screening methods useful for detecting bacteria and leukocytes in urine.

Detection of pyuria

Pyuria often indicates urethritis, cystitis, or pyelonephritis. Detection of leukocytes may be performed by a urine dipstick or microscopic examination of a wet mount of a urinary sediment. At least five fields should be examined, and each leukocyte seen per high-power field ($\times 400$) represents approximately 5 to 10 cells per microliter of urine. In this way, 5 to 10 leukocytes per high-power field in the sediment is the upper limit of normal, representing at least 50,000 cells/mL. If a more precise method is required for cell quantitation, cells in unconcentrated urine can be counted using a hemocytometer chamber. The presence of more than 8 leukocytes/ μ L indicates significant pyuria.

Case check 37.4

Urinalysis and microscopic examination can aid in the diagnosis of UTIs by detecting leukocyte esterase and the number of leukocytes, which can add to the significance of the bacterial colony count. The presence of bacteriuria and pyuria usually indicate infection. Microscopic examination can help with the preliminary detection of infectious etiologies to guide early therapy.

Detection of fungi and mycobacteria

First morning specimens are recommended, and large volumes (10 mL to 50 mL) give best results. Urine should be centrifuged at $2000 \times g$ for 10 minutes, especially when deep mycoses are suspected. Yeast cells can usually be readily identified by microscopic analysis, but the cells of other fungi may be difficult to discern because of their varied size and unique forms. For these reasons, an examination of a wet mount

should be scanned at $100\times$ and $400\times$ magnifications before using $1000\times$. If fungi are suspected clinically or from microscopic analysis, a smear of the sediment can be stained with calcofluor white and examined using fluorescent microscopy. This stain binds to chitin present in the cell walls of fungi and makes visualization easier. Because calcofluor white also binds to cellulose, which chemically resembles chitin, cotton swabs should not be used because they too will fluoresce. Quantitative colony counts, as performed for bacteria, are not useful for fungi. In pyelonephritis studies from the 1970s, researchers did not find a correlation between infections confirmed by renal biopsy and colony counts.

Automated urine screening methods

Table 37.7 summarizes various automated screening methods used for bacterial detection in urine samples. Such systems may be expensive, frequently require batching of specimens (therefore causing time delays), and have not been adequately evaluated for their efficacy in detecting low-grade bacteriuria and funguria.

Molecular screening and identification methods

Molecular methods have the advantage of providing faster microbial identification than traditional culture techniques but are incapable of discerning organism viability. This technique shows promise with identification of the most common infectious organisms, such as *E. coli*, but is not useful for uncommon infectious agents. Furthermore, predicting antimicrobial susceptibility by polymerase chain reaction (PCR) can only account for resistance mechanisms that are genetically based and previously identified. Mechanisms such as chemical changes to the cellular outer membrane layer would be undetected by most molecular techniques and provide a discrepancy for antimicrobial susceptibility between PCR and culture techniques. Another drawback to molecular analysis is if multiple pathogens are detected, it is difficult to

Table 37.7 Automated screening methods, principle of assay, and threshold of detection for urinary tract infections

Automated method	Principle	Threshold of detection (CFU/mL)
Bioluminescence	Detects bacterial ATP using enzymatic bioluminescent reaction of ATP with luciferin and luciferase	$>10^4$ – 10^5
Photometry	If a significant number of organisms are present in the urine specimen, they will grow in the medium to a concentration detectable by photometry (i.e., light scattering)	$>10^4$ – 10^5
Flow cytometry	Detects and quantifies bacteria, WBCs, and RBCs with color-coded scatter diagrams	$>10^3$
PCR	Detects specific nucleic acid sequences that were selected to be unique to the organism or groups of organisms common in UTIs	Varies depending on targets used and assay specifications

ATP, Adenosine triphosphate; CFU, colony-forming unit; PCR, polymerase chain reaction; RBC, red blood cell; UTI, urinary tract infection; WBC, white blood cell.

determine if this is a true polymicrobial infection or contamination. Only culture or quantitation of each pathogen target relative to one another can determine this.

Rejection criteria

It is imperative that the laboratory establishes, in concert with various medical services, the criteria for obtaining optimal specimens. Specimens may be rejected because of an inadequate or inappropriate method of collection or transport. These criteria demand strict adherence to guidelines for the collection and transport of specimens. If the specimen does not meet these tailored guidelines for each institution, it is incumbent on the laboratory to inform (as soon as possible) the service, health care provider, or both about the inadequacy of the specimen and the fact that the specimen will not be processed. Antimicrobial therapy may not have been initiated, and a better specimen might still be collected. Samples to be rejected include 24-hour urine specimens and Foley catheter tips.

Culture for causative agents of urinary tract infections

Methods for culture differ among different laboratories. Generally, routine urine culture should include plating onto one gram-negative selective medium (e.g., MacConkey or eosin–methylene blue agar) and one nonselective medium (sheep blood agar). Columbia colistin–nalidixic acid blood agar can inhibit gram-negative rods and allow gram-positive organisms to grow, but a comparison of the gram-negative differential to nonselective plate can generally yield the same information without the need for an additional medium. Chromogenic agar can be useful to identify and distinguish organisms in mixed cultures. These include CPS ID3 (bioMérieux, Durham, NC), Remel Spectra UTI chromogenic medium (Thermo Fisher Scientific, Waltham, MA), and BBL CHROMagar Orientation (BD Diagnostic Systems, Sparks, MD).

Calibrated loops of 0.001 mL (1 μ L) or 0.01 mL should be used to quantify the number of bacteria present in the urine sample. Use of 0.01 mL can detect as few as 10^2 CFUs. The urine specimen should be mixed thoroughly, and the calibrated loop should be inserted vertically as a more horizontal position can increase the volume beyond calibration. The loop should be redipped for each plate inoculated.

Streaking one plate for quantitation using a T-streak method and another for isolation using a traditional quadrant streak may help quantitate and isolate colonies for further work and lower the possibility of subculture to additional media before identification and antimicrobial susceptibility testing is required.

Plates should be incubated for at least 16 hours, but preferably for 18 to 24 hours at 35° C. The culture protocol should be able to detect the most common pathogens, such as Enterobacterales, and species of *Staphylococcus*, *Pseudomonas*, and *Enterococcus*. Antimicrobial susceptibility testing should be performed on organisms with significant colony counts or from symptomatic patients who have three or fewer morphologically distinct colony types. More than four colony types, especially when they are similar in abundance, suggests contamination and should be reported as such.

Asymptomatic bacteriuria

If the patient is asymptomatic, immediate antimicrobial therapy is not necessary unless the patient is pregnant or undergoing GU surgery. Identification and susceptibility testing of isolates can be achieved by conventional or automated methods. The colony count should accompany any positive culture result to indicate the diagnosis of ASB. Because the organisms most frequently identified include *E. coli* and other rapidly growing members of the order Enterobacterales, 24 hours of incubation at 35° C is sufficient.

Pyelonephritis

Urine specimens submitted from patients suspected of having pyelonephritis generally contain high colony counts of bacteria. Microscopic examination of urine for leukocytes and bacteria might quickly provide therapeutically useful information. Because the antimicrobial susceptibilities of the responsible organisms are variable and unpredictable, culture should be designed for optimal recovery and antimicrobial susceptibility testing.

Lower urinary tract infections

Specimens from patients with symptoms of L-UTI should be processed in the same manner as for suspected cases of pyelonephritis. If there is significant pyuria in a patient who is symptomatic and no recognized urinary pathogen is

detected, then *C. trachomatis*, *Mycoplasma* spp., *U. urealyticum*, *N. gonorrhoeae*, or *M. tuberculosis* should be suspected. For gonococcus and chlamydia, first-morning voided urine (not midstream urine) is recommended. Urine PCR is available to detect *N. gonorrhoeae* and *C. trachomatis*.

Suprapubic aspirates

Suprapubic aspirates might contain bacteria likely to be present in low numbers and may include anaerobic species. Such specimens should be routinely inoculated on a blood agar plate, MAC agar plate, and trypticase soy broth (TSB) for up to 48 hours. For recovery of *G. vaginalis*, chocolate agar is acceptable. If growth is observed in the lower half or less of the TSB tube and little or no growth is present on the aerobic plates, an anaerobe may be considered. Anaerobic bacteria are recovered in approximately 1% of cases and therefore need only be sought after consultation.

Catheterized specimens

Urine specimens obtained by straight catheterization, bilateral ureteral catheterization, bladder washout, or ileoconduits require inoculation of agar plates for maximum recovery.

Case check 37.5

Urine can be collected by several methods. The simplest is voided midstream, which is also most likely to be contaminated. Catheterized specimen collection is more invasive, but specimens are less likely to be contaminated. The definitive method to obtain an uncontaminated urine specimen is suprapubic aspiration, which is also the most invasive.

Prostatic secretions

The causative agent of acute prostatitis is usually recovered from catheterized specimens, which should be cultured in the same manner as for specimens from symptomatic male patients. In cases of chronic prostatitis, prostatic secretions are submitted as well as urethral urine and midstream-voided urine specimens obtained before and after prostatic massage. Quantitative cultures are necessary for proper interpretation.

Historically, yeasts have been detected within 24 hours, but certain species may need 48 hours of incubation. When isolated, the numbers of yeasts should be reported. Low colony counts may be just as significant as high colony counts.

Interpretation of results

Routine workup of isolates and susceptibility testing must be tailored according to the patient and the specimen type submitted. Fig. 37.4 shows a flow diagram that considers three features that should be considered in all UTIs:

- Colony count of a pure or predominant organism
- Measurement of pyuria
- Presence or absence of symptoms (e.g., dysuria and urinary frequency)

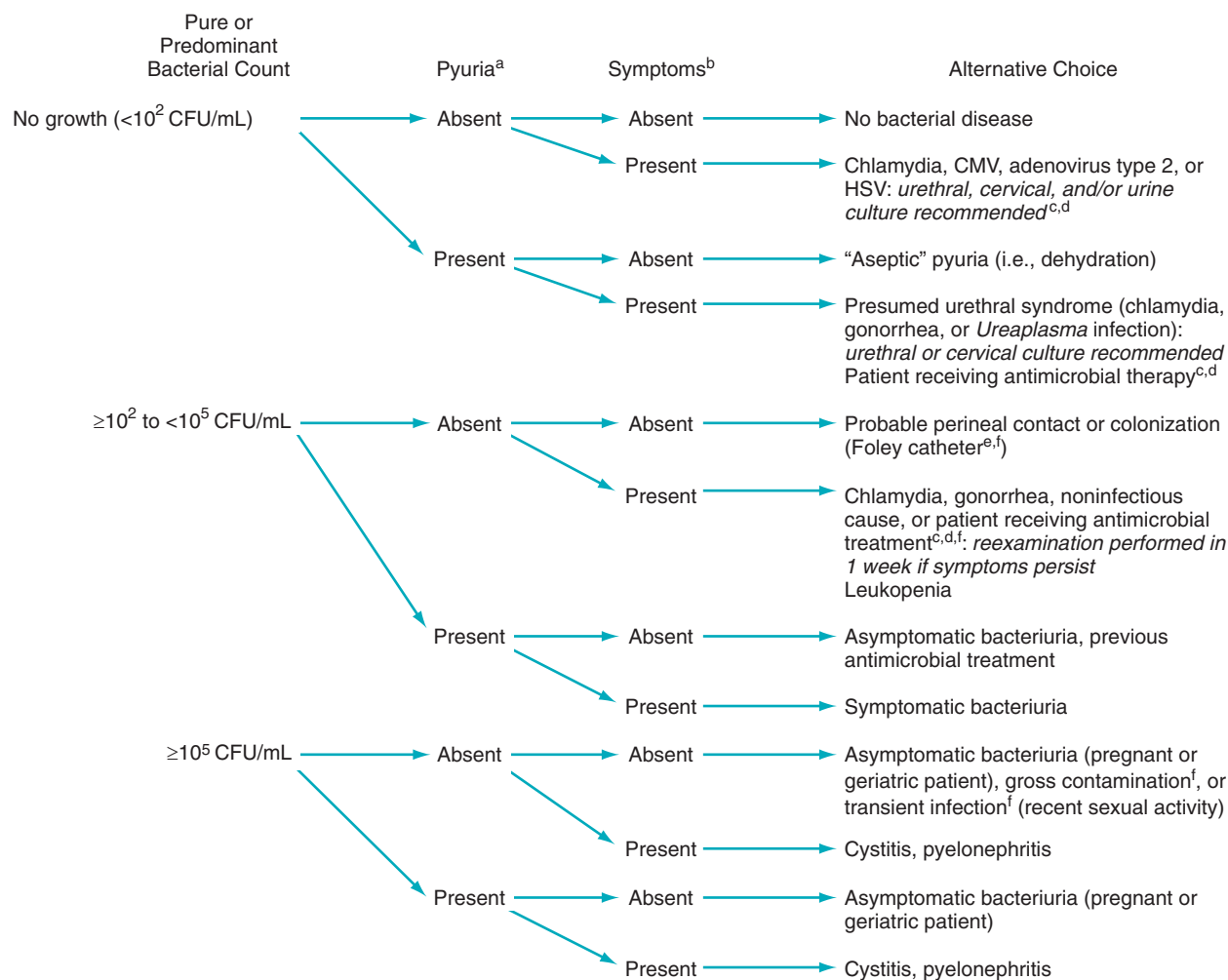
It is important to recognize, however, that no one scheme fits all situations. Fig. 37.4 is organized into a cascade scheme using a dichotomous key, which allows for 12 clinical categories, depending on knowledge of symptoms and health care provider input. Historically, most guidelines suggested that laboratory evaluation and culture setup should depend on previous knowledge of symptoms. Because this is often unrealistic, the schema presented allows the final differentiation to be made by the health care provider, based on his or her knowledge of the patient's signs and symptoms. Furthermore, if the patient is receiving antimicrobial therapy, microscopic examination, WBC analysis, and culture results might not agree. Finally, quantitation of organisms and WBCs by urinalysis of a centrifuged specimen has no comparative value for the leukocyte esterase and bacteria measurement in microbiological study, which is performed routinely on a noncentrifuged urine specimen. Fig. 37.4 addresses acute urethral syndrome and the recognition that cystitis and urethritis are clinically difficult to differentiate, particularly in female patients. It is imperative that health care providers recognize that routine urine cultures do not include the isolation and identification of *C. trachomatis*, *N. gonorrhoeae*, or *U. urealyticum*.

Given the high propensity of negative cultures sent to the laboratory ($\approx 50\%$), reevaluation of the patient with a negative culture result may require consideration of an STI. Table 37.8 lists guidelines for the interpretation of urine culture results and suggests the subsequent workup. In summary, these guidelines recommend the following, keeping in mind that cost-effective strategies might define different algorithms for inpatient and outpatient cases:

- Multiple uropathogens (i.e., three or more) in a specimen indicates probable contamination.
- One or two significant uropathogens present ($\geq 10^5$ CFU/mL) should be routinely identified. Susceptibility tests should be performed for inpatients. Outpatient cases may use a different algorithm that does not call routinely for susceptibility tests; rather, it emphasizes empiric selection based on antibiograms. However, some centers may perform susceptibility testing on all urine culture isolates in the setting of increasing drug resistance.
- One or two uropathogens present in small numbers ($\geq 10^2$ to $<10^5$ CFU/mL) should be routinely identified if the clinical situation warrants, such as in acute urethral syndrome or cases of previous antimicrobial therapy.

Susceptibility reporting

With the growing number of emerging uropathogens and simultaneous increase in newer antimicrobial agents, it is mandatory that laboratories use standardized methods and report only appropriate agents for treating UTIs. Antimicrobial agents approved by the U.S. Food and Drug Administration (FDA) for routine testing and reporting by clinical microbiology laboratories for urinary tract isolates are listed as group U supplemental for urine only in the Clinical and Laboratory Standards Institute (CLSI) M100 document, which is updated annually. The CLSI document provides interpretations of



^aLeukocyte esterase (+); equivalent to 5 WBCs/hpf.

^bClinical dysuria and frequency.

^cIf patient is receiving antimicrobial treatment, the result of the Gram stain, WBC analysis, and culture may not agree.

^dQuantitation of organisms and WBCs by urinalysis of a centrifuged specimen is of no comparative value for the measurement of leukocyte esterase and bacteria done by microbiological study, which is performed routinely on a noncentrifuged specimen.

^eInterpretation for indwelling catheter has not been established.

^fPlates held for 72 hours for consultation.

Fig. 37.4 Interpretation of urine culture results using an algorithm based on bacterial colony count, pyuria, and symptoms. CMV, Cytomegalovirus; hpf, high-power field; HSV, herpes simplex virus; WBC, white blood cell.

antimicrobial susceptibilities based on both the minimum inhibitory concentration (MIC) method and the disk diffusion methods. Because the interpretation of MIC or disk diffusion results can vary based on additional scientific and case study analysis, laboratory directors must make yearly decisions about whether they will update the CLSI standard or retain the FDA-approved values. Refer to [Chapter 13](#) for more information on antimicrobial susceptibility testing. Several laboratories have tailored susceptibility testing according to the needs of the clinical setting: outpatient versus inpatient, pediatric versus adult, and ICU versus non-ICU. It is important to remember that attainable antimicrobial blood levels and urine levels are often different; this will have an impact on the

interpretation of some semi-quantitative susceptibility results or the MIC of inhibitory antimicrobial agents.

Urinary tract infection antibiograms

Historically, one of the primary functions of the clinical microbiology laboratory has been to measure antimicrobial resistance trends. This was generally accomplished by using an annual antibiogram that established cumulative percentage susceptibilities for selected UTI bacteria–antimicrobial combinations. Today, this is even more important and requires focused antibiograms that tailor these historical resistance fingerprints to selected patient locations, inpatient versus

Table 37.8 Guidelines for interpretation of urine culture results and subsequent workup

Significant colony count (CFU/mL) ^a	Symptoms, clinical disease, or patient population ^b	Urine source	Number of organism types isolated	Laboratory workup suggested (inpatient) ^c
$\geq 10^4$ $\geq 10^3$ ^b		CC CA	None	None ^d
$\geq 10^4$	Pediatric	Suprapubic	≤ 2 organisms by anaerobic culture	ID and AST
$\geq 10^4$	Symptomatic female, urethritis	CC	Pure culture	ID and AST
$\geq 10^4$	Symptomatic male, prostatitis	CA CA	≤ 2 organisms Pure culture	ID and AST ID and AST
$\geq 10^4$		Bladder washout		ID and AST
$\geq 10^4$	Cystitis, pyelonephritis	CC	Pure culture Two or three organisms Three organisms	ID and AST Q and SID Q and M or Q and GS

CA, Straight catheterized; CC, clean-catch voided; CFU, colony-forming unit; ID and AST, perform identification and antimicrobial susceptibility testing; Q and GS, quantitate and report Gram stain morphotypes; Q and M, quantitate total amount of bacteria and report as "mixed urethral microbiota"; Q and SID, quantitate and perform sight identification, identification and sensitivity not indicated, hold plates for 72 hours.

^aInoculation of 0.01 mL of urine is required to detect 10^2 CFU/mL, however, at least 10 colonies on the plate are required for significance. Use of a 1- μ L loop means reporting of 10^4 CFU/mL or a 10- μ L loop for reporting of 10^5 CFU/mL or greater colony counts.

^bSee Table 37.1 for a description of clinical diseases, symptoms, and patient population.

^cWorkup required. Any yeast may be quantitated and reported, regardless of number; $>100,000$ needed to identify to species.

^dSee figures and text for suggested comments and educational information helpful to health care providers.

outpatient status, disease type, and patient age. Most importantly, if the laboratory has a large enough volume of isolates to enable reassessment of an antibiogram more frequently than annually, it should be a consideration to help clinicians choose empiric therapy for inpatient and long-term care settings. Outpatient urine isolates often have a stable and predictable pattern.

POINTS TO REMEMBER

- UTIs occur frequently, especially in women; 50% of all women will have a UTI in their lifetime.
- The pathogenesis and course of a UTI depends on the organs involved and the patient's state of health.
- L-UTIs involve the bladder (cystitis), urethra (urethritis), or prostate (prostatitis). Symptoms can include dysuria, hematuria, and changes in urinary frequency.
- U-UTIs involve the kidneys (pyelonephritis) or ureters (ureteritis). Symptoms can include fevers, chills, night sweats, nausea, vomiting, flank pain, and costovertebral angle tenderness.
- Common causative agents of UTIs include coagulase-negative staphylococci, *E. coli*, *Klebsiella* spp., other Enterobacteriales, and enterococci. *Pseudomonas* spp., *Proteus* spp., and *Candida* spp. are also seen, especially in healthcare- or catheter-associated UTIs.
- Urinalysis and culture are important components in the evaluation of UTIs. The presence of bacteriuria and pyuria indicates infection.
- Results of a urinalysis dipstick (i.e., positive leukocyte esterase and nitrites) often will result in a reflex to a urine culture by most clinical laboratories.
- Interpretation of urine cultures depends on several factors including number and types of isolates, colony counts, type of specimen, and patients' history.

LEARNING ASSESSMENT QUESTIONS

1. Which scenario pertaining to urine cultures is most likely to be clinically significant?
 - a. Asymptomatic pregnant female patient with bacteriuria, pyuria, and 10^3 CFU/mL of *E. coli* in a voided urine specimen
 - b. Asymptomatic female patient with bacteriuria, no pyuria, and 10^4 CFU/mL of *E. coli* in a voided urine specimen
 - c. Asymptomatic older adult male patient with 10^5 CFU/mL of *S. epidermidis* and enterococci in a urine sample collected from a Foley catheter
 - d. Symptomatic male patient with 10^5 CFU/mL of *E. coli*, *S. epidermidis*, and *P. mirabilis*
2. Which collection method is most likely to result in urine culture contamination?
 - a. Suprapubic aspirate
 - b. Collection from a urine catheter port after catheter placement
 - c. Clean catch from a male patient
 - d. Urine collected from a Foley bag
3. If a blood culture and urine culture are positive with the same organism, what is the likely route of the urinary tract infection?
 - a. Ascending route
 - b. Lymphatic route
 - c. Hematogenous route
 - d. These are unrelated, and route of infection cannot be connected
4. In which of the following patient populations are you most likely to culture *Proteus* spp. from urine?
 - a. 2-year-old female patient
 - b. 16-year-old sexually active female patient
 - c. 85-year-old catheterized male patient
 - d. Renal transplant patient

5. A culture was inoculated at 10:00 PM, and a newly hired day shift laboratory scientist removed the lid of the culture plate briefly and read the culture at 11:00 AM the next day. This culture was noted as no growth and returned to the incubator for the next day. The following day, an experienced day shift laboratory scientist read the culture as contaminated and reported the result as such. Which of the following is the most likely explanation of why the initial read showed no growth and later appeared contaminated?
 - a. The experienced day shift laboratory scientist misread the plates.
 - b. The first culture was read too early to determine growth status.
 - c. The plates became contaminated because the lid of the culture was removed briefly.
 - d. The newly hired laboratory scientist was not experienced enough to see growth on the plate and erroneously missed the growth.
6. A clean-catch midstream urine sample is received in the clinical laboratory at 6:00 PM. The microbiology laboratory will not be staffed until 7:00 AM the next day. At which temperature should the urine sample be stored?
 - a. 35° C
 - b. 30° C
 - c. 4° C
 - d. -20° C
7. Which of the following is an acceptable practice for urine transportation when a culture will be performed?
 - a. Sending it to the laboratory frozen
 - b. Sending a specimen on ice 3 days after collection
 - c. Sending urine in a sterile collection cup 4 hours after collection at room temperature
 - d. Collecting and immediately transferring the sample to a preservation tube so it will arrive to the laboratory within 24 hours
8. A patient presents to the emergency department with symptoms of a UTI. Your laboratory information system reveals that the patient had a UTI with *E. coli* 3 months earlier. What is the next step to take?
 - a. Do not set up a culture as the health care provider is likely already treating the patient.
 - b. Do not set up a culture and refer the health care provider to the previous urine culture.
 - c. Set up cultures, and if *E. coli* is isolated, consider making a note to the health care provider that previous infection grew the same organism.
 - d. Set up cultures, and if *E. coli* grows again, disregard the result because it is likely a contaminant.
9. Which one of the following organisms is most likely to be a contaminant in a urine culture?
 - a. *Candida albicans*
 - b. *Klebsiella oxytoca*
 - c. *Neisseria gonorrhoeae*
 - d. *Staphylococcus epidermidis*
10. A health care provider must decide on the best antimicrobial agent to treat a patient before the antimicrobial susceptibility test results are available. Which of the following is *not* a useful guide in deciding the empiric therapy?
 - a. Past patient history of infection and treatment
 - b. The least expensive antimicrobial agent in the pharmacy
 - c. Antibigram published each year by the microbiology laboratory
 - d. Understanding of the patient demographics and likely organisms involved
11. How would the urine culture described in the Case in Point be worked up and reported?
12. Compare the route of urinary tract infection presented in the Case in Point versus common gram-negative bacteria.
13. What is the significance of the patient's clinical symptoms and the positive blood culture results?
14. What is the difference between a single-episode UTI and a recurrent UTI?
15. What is the value of a urinalysis algorithm leading to microbial culture?
16. What is the optimal incubation period for routine urine culture?
17. What may occur if routine urine cultures are incubated for longer than the optimal period?
18. What is the definition of a contaminated urine culture?
19. Why is it important for health care providers to reevaluate negative urine culture results on specimens from symptomatic patients?
20. How would your list of potential urinary tract infection isolates differ among the following patients: a young, sexually active female patient; a middle-age male patient; an older adult female patient; and a patient who has had a recent kidney transplant?
21. A patient with diabetes who underwent kidney transplantation 6 weeks ago develops fever, chills, nausea, vomiting, and dysuria. Which laboratory tests should be performed to aid in the diagnosis of this patient's condition?

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Genital infections and sexually transmitted infections

Denene Lofland

CHAPTER OUTLINE

Urethritis, 945

- Causes, 945
- Epidemiology, 945
- Clinical manifestations, 946
- Laboratory diagnosis, 947
- Treatment, 948

Cervicitis, 948

- Causes, 948
- Epidemiology, 949
- Clinical manifestations, 949
- Laboratory diagnosis, 949
- Treatment, 949

Vulvovaginitis, 949

- Causes, 949
- Bacterial vaginosis, 950
- Trichomoniasis, 951
- Candidiasis, 952

Genital ulcer disease, 953

- Causes, 953
- Genital herpes, 954
- Syphilis, 956
- Chancroid, 959
- Lymphogranuloma venereum, 960
- Donovanosis, 962

Acquired immunodeficiency syndrome, 963

- Causes, 963
- Epidemiology, 964
- Clinical manifestations, 964
- Laboratory diagnosis, 966
- Treatment, 967

Other sexually transmitted diseases, 967

- Genital warts, 968
- Viral hepatitis, 969

Pelvic inflammatory disease, 970

Molluscum contagiosum, 971

Epididymitis, 971

Proctitis, 971

Bibliography, 972

OBJECTIVES

After reading and studying this chapter, you should be able to:

1. Describe the pathogenesis of the following diseases:
 - Urethritis
 - Cervicitis
 - Vulvovaginitis
 - Genital ulcer disease
 - Acquired immunodeficiency syndrome
2. Recognize the common clinical manifestations, complications, and treatment of pelvic inflammatory disease.
3. Discuss how the advent of molecular testing has affected the identification of sexually transmitted infections.
4. Justify the rationale for offering the human papillomavirus vaccine to those between 9 and 26 years of age.
5. Interpret an acute hepatitis panel and testing results specific for hepatitis B and C.
6. Differentiate the signs, symptoms, and causative agents of gonococcal and nongonococcal urethritis.
7. Compare the laboratory testing methods used to presumptively diagnose or confirm a urethral infection.
8. Justify performing screening tests for women who are sexually active but have no signs or symptoms of infection.
9. Identify potential long-term effects of an untreated infection.
10. Compare the signs and symptoms of bacterial, fungal, and parasitic forms of vulvovaginitis.
11. Describe the expected results of diagnostic tests that can be performed by the health care provider to determine the causative agent of vulvovaginitis.

12. Correlate the signs, symptoms, and laboratory tests required to differentiate among various genital ulcerative diseases.
13. Explain the requirement for performing screening and confirmatory tests for syphilis.
14. Interpret the various expected results of laboratory tests to differentiate between active and latent syphilis.
15. Determine the laboratory test results required to diagnose human immunodeficiency virus (HIV) infection (presumptive or confirmed) in an individual.
16. Evaluate the long-term effects of HIV infection on an individual's immune system.
17. Justify the recommended laboratory tests to be performed after HIV infection has been diagnosed in an individual.

KEY TERMS

Acquired immunodeficiency syndrome (AIDS)	Highly active antiretroviral therapy (HAART)
Bacterial vaginosis (BV)	Human immunodeficiency virus (HIV)
Balanitis	Human papillomavirus (HPV)
Cervicitis	Lymphogranuloma venereum (LGV)
Chancere	Molluscum contagiosum
Chancroid	Nongonococcal urethritis (NGU)
Chlamydia	Nontreponemal antibody tests
Condylomata accuminata	Pelvic inflammatory disease (PID)
Condylomata lata	Proctitis
Donovan bodies	Salpingitis
Epididymitis	Syphilis
Genital herpes	Treponemal antibody tests
Gonorrhea	Trichomoniasis
Granuloma inguinale (donovanosis)	Urethritis
Gummas	Vulvovaginal candidiasis
Hepatitis	

Case in point

A 19-year-old college freshman presents to the student health center complaining of a burning sensation when he urinates. After a recent argument with his girlfriend, the patient spent the weekend on a road trip with several friends, where he indulged in a few casual encounters of unprotected sex. A cloudy watery penile discharge is noted. Direct Gram-stained smear demonstrates a moderate number of white blood cells (WBCs), and no organisms are seen. A urethral swab is submitted to the laboratory for molecular testing, and blood is drawn for **human immunodeficiency virus (HIV)** testing. The patient's health care provider questions him about sexual contacts. The student mentions that his girlfriend, who drove him to the health center, is in the waiting room. The girlfriend, in a private interview, reports not having any genital discomfort or dysuria. The couple does not use condoms, but the girlfriend is taking birth control medication. She has not received the **human papilloma-virus (HPV)** vaccine. She does mention that she woke up that morning with a sore throat and has been taking throat lozenges. Inspection of her throat reveals slight redness and irritation. No throat specimen is submitted for testing. Both the patient and his girlfriend are provided with oral antimicrobial agents, and he is encouraged to notify all of his sexual partners.

Issues to consider

After reading the patient's case history, consider:

- The significance of the direct Gram-stained smear findings of the exudate showing a moderate number of WBCs and no organisms seen
- Why the patient should notify all of his sexual partners
- Whether or not the girlfriend could be infected, even though she was found to have no current signs or symptoms
- Which signs and symptoms would be noted for syphilis, chancroid, and herpes
- If an HIV screening test for this patient would be indicated and be of value

Genital infections and sexually transmitted infections (STIs) are caused by organisms normally present in the reproductive tract or introduced from the outside during sexual contact. STIs are spread primarily through intimate person-to-person sexual contact, including vaginal intercourse, oral sex, and anal sex. Several STIs, in particular **acquired immunodeficiency syndrome (AIDS)**, **chlamydia**, and **gonorrhea**, can also be transmitted from mother to child during pregnancy and childbirth. HIV, the causative agent of AIDS, can be transmitted through blood products and tissue transfer. There are more than 30 different sexually transmissible bacteria, viruses, epizoa, fungi, and parasites. Use of the term *sexually transmitted diseases* (STDs) denotes a diverse group of infectious diseases with a wide variety of pathogens and wide-ranging clinical manifestations (Table 38.1). The term

Table 38.1 Sexually transmitted diseases and their causative agents

Disease	Agent(s)
AIDS	HIV-1, HIV-2 ^a
Bacterial vaginosis	<i>Gardnerella vaginalis</i> , <i>Mobiluncus</i> spp., and so on
Chlamydia	<i>Chlamydia trachomatis</i>
Chancroid	<i>Haemophilus ducreyi</i>
Cytomegalovirus disease	Cytomegalovirus
Genital warts	Human papillomavirus
Gonorrhea	<i>Neisseria gonorrhoeae</i>
Donovanosis (granuloma inguinale)	<i>Klebsiella granulomatis</i>
Leukemia, lymphoma	HTLV-I, HTLV-II
Lymphogranuloma venereum	<i>Chlamydia trachomatis</i>
Molluscum contagiosum	Molluscum contagiosum virus
Nongonococcal urethritis	<i>Mycoplasma genitalium</i> , <i>Ureaplasma urealyticum</i>
Pubic lice	<i>Phthirus pubis</i>
Scabies	<i>Sarcoptes scabiei</i>
Syphilis	<i>Treponema pallidum</i> subsp. <i>pallidum</i>
Trichomoniasis	<i>Trichomonas vaginalis</i>

^aHIV-2 is found predominately in sub-Saharan Africa.

AIDS, Acquired immunodeficiency syndrome; HIV, human immunodeficiency virus; HTLV, human T-cell leukemia virus.

sexually transmitted infections (STIs) is more commonly used because it has a broader range of meaning; a person may be infected and may potentially infect others without showing signs of disease.

STIs continue to be a major health threat in the United States and worldwide. According to the World Health Organization (WHO), 376 million new cases of curable STIs (syphilis, gonorrhea, chlamydia, and trichomoniasis) occur throughout the world every year. Additionally, more than 1 million STIs are acquired daily worldwide. In developing countries, STIs and related complications rank in the top five in disease categories for individuals seeking health care. Up to 70% of women and a large percentage of men may show no signs or symptoms of gonococcal or chlamydial infections and therefore do not receive treatment or counseling. Individuals who are unaware of their infection status can thus perpetuate the spread of disease through each new contact.

Without treatment, there are critical implications of STIs for the reproductive, maternal, and newborn health. Because developing countries often have limited resources and testing capabilities, the WHO has developed a syndromic approach to diagnose STIs and treat patients presenting with consistently recognized signs and symptoms of an STI. Although not perfect, this syndromic approach is more accurate than diagnosis based on clinical tests alone. In the United States, the Centers for Disease Control and Prevention (CDC) estimated in 2018 that 26 million new STIs were diagnosed, costing the U.S. health care system \$16 billion in direct medical costs. Current reviews of mandatory reporting data indicate continuing annual increases for syphilis, gonorrhea, and chlamydia. Unfortunately, no trending data are available for STIs that do not require reporting.

The CDC's 2020 surveillance study reports that the increase in 2018 represents a disturbing 185% increase in the congenital syphilis case rate relative to 2014. During this period, a corresponding 165.4% increase in primary and secondary syphilis in women of childbearing age was observed. From 2017 to 2018, newborn deaths from syphilis increased 22%.

The national congenital syphilis rate of 57.3 cases per 100,000 live births in 2020 represents a 15% increase relative to 2019 and a 254% increase relative to 2016. These increases mirror increases in syphilis among reproductive-aged women. During 2019–2020 the rate of primary and secondary syphilis increased 24% among women aged 15 to 44 years. In 2020 there were 5726 cases of syphilis (all stages) diagnosed among pregnant women, an increase of 16% from 2019.

A great disparity of infection rates exists among specific age ranges and among some ethnic and racial groups. Approximately 10,000 teenagers are infected by STIs daily, which averages to about 1 every 8 seconds. About one half of the new infections in the United States occur in persons 15 to 24 years of age, many of whom may develop lifelong health issues. According to the 2020 Sexually Transmitted Diseases Surveillance by the CDC, almost two thirds (61%) of all reported chlamydia cases were among persons aged 15 to 24 years. Gonorrhea and syphilis cases were highest among 20- to 24-year-old men and women.

Disparities also exist among some ethnic and racial groups. The rates of chlamydia, gonorrhea, and syphilis were

5.6 times, 7.7 times, and 4.7 times higher, respectively, among Blacks than among Whites. Much of this may be caused by poorer living conditions, lack of convenient health care resources, or cultural barriers.

Men who have sex with men (MSM) are disproportionately affected. They account for 54% of the syphilis cases, and among these men, 42% have HIV. The incidence of antibiotic-resistant gonorrhea is greater compared with women and men who have sex with women only. The CDC continues to invest resources in prevention efforts to reduce risk behavior and increase STI and HIV testing among populations at greatest risk.

Currently, the CDC recommends annual chlamydia and gonorrhea screening for sexually active women younger than 25 years of age and for older women with risk factors (e.g., new or multiple sexual partners or residing in communities with a high burden of disease). Pregnant women should also be tested for syphilis, HIV, and hepatitis B. Annual screening of MSM for syphilis, gonorrhea, and chlamydia is recommended. HIV screening is recommended at least once for everyone between 13 and 64 years of age and annually for anyone not practicing safe sex or sharing injection drug supplies. Although these screening and prevention programs have been proven cost-effective and beneficial to the public, more has to be done to stem the tide because approximately 50% of those who should be screened are actually tested. Unfortunately, many people still engage in risky sexual behavior, even after receiving a diagnosis of an STI, which can be correlated with increased drug use.

In 2018, four of the top 10 reportable diseases in the United States were STIs (chlamydia, gonorrhea, syphilis, and HIV infection; [Table 38.2](#)). STIs have diverse causative agents, signs and symptoms, prognoses, and methods for laboratory testing. This chapter will discuss common symptoms of STIs and review exudative infections (gonorrhea, chlamydia, vulvovaginitis), ulcerative infections (syphilis, chancroid, genital herpes), HIV infection, and other STIs.

Table 38.2 Top 10 reportable diseases in 2018

Disease, organism	Cases reported (/100,000)
Chlamydia	537.54
Gonorrhea	178.32
Syphilis—total, all stages	35.16
Campylobacteriosis	21.46
Salmonellosis	18.64
Coccidioidomycosis	11.58
Lyme borreliosis	10.34
Human immunodeficiency virus	10.09
<i>Streptococcus pneumoniae</i> , invasive disease	8.14
Giardiasis	6.06

From Centers for Disease Control and Prevention. MMWR: Summary of Notifiable Infectious Diseases: Annual Notifiable Diseases Table 2018.

Urethritis

Causes

Urethritis is the most common STI syndrome recognized in men and is often seen in women with coinciding cervicitis. **Urethritis** is an inflammation of the urethra, which can be caused by mechanical injury (catheterization), chemical irritation (antiseptics), or infectious disease. Infectious urethritis is usually associated with organisms that cause STIs, such as *Neisseria gonorrhoeae* and *Chlamydia trachomatis*, which clinically manifest as exudates. Cases can be divided into two types based on causation: gonococcal urethritis and **nongonococcal urethritis (NGU)**. In males, gonococcal urethritis can be presumptively diagnosed with direct Gram-stained smears of urethral exudates, which will demonstrate WBCs with gram-negative intracellular diplococci suggestive of *N. gonorrhoeae*. Because women have normal biota resembling *N. gonorrhoeae*, a similar finding in endocervical exudates must be supported by either culture or a nonculture assay.

NGU, which can have infectious and noninfectious causes, is diagnosed similarly when inflammation (WBCs) is detected with no organisms seen on direct Gram-stained smears. Most NGU cases cannot be attributed to any specific agent. Infection with *C. trachomatis* is the most common cause of NGU (15% to 55% of cases) and is the most commonly reported infectious disease in the United States. Less frequent causes of NGU include *Ureaplasma urealyticum*, *Mycoplasma genitalium*, *Trichomonas vaginalis*, herpes simplex virus (HSV), and adenovirus. Gonococcal urethritis and NGU infections are not mutually exclusive. Many patients are often co-infected with *N. gonorrhoeae* and *C. trachomatis*, with rates as high as 20% in men and 42% in women.

Case check 38.1

The two main causative agents of urethritis are *N. gonorrhoeae* and *C. trachomatis*. In males presenting with a purulent exudate or discharge, direct Gram-stained smear of the discharge demonstrates many polymorphonuclear cells with intracellular gram-negative diplococci, which are a strong predictor of gonococcal infection. Generally, WBCs noted in a urethral exudate are suggestive of an infection. In the Case in Point, the patient had a discharge that demonstrated WBCs but no organisms. Clearly, he had urethritis, but without further testing it is impossible to determine the causative agent. Some patients can have a co-infection with *N. gonorrhoeae* and *C. trachomatis*. In general, most patients presenting with symptoms of urethritis are treated with antimicrobials effective against both organisms. The patient's girlfriend was also given antimicrobials despite not having any symptoms.

Epidemiology

Gonorrhea is the second most commonly reported infectious disease in the United States. According to the CDC, 677,769 cases of gonorrhea were reported in 2020 in the

United States. The overall rate of reported gonorrhea increased 5.7% during 2019–2020. However, the CDC estimates that only 50% of new infections are reported, indicating a high probability that over 1,000,000 people contract new gonorrheal infections each year. Rates of reported gonorrhea have increased 111% since the historic low in 2009, although there was a slight decrease in 2013, followed by a yearly increase during 2013–2020 (Fig. 38.1). Rates have been higher among men compared to women since 2013 when cases were identified in both men who have sex with men (MSM) and men who have sex with women only. However, during 2019–2020, the CDC reported that rates among women were greater (15%) than among men (6.6%), suggesting the differences in diagnosing and reporting of cases among MSM in 2020.

The highest incidence of gonorrhea continues to be found among sexually active adolescents and young adults from 15 to 24 years of age. Worldwide, gonorrhea rates have also continued to increase over the past 20 years, with an estimated 86.9 million new cases reported annually. Many of these infected individuals are asymptomatic (approximately 80% of women, 10% of men), which is a cause of concern with regard to treatment and control, especially because individuals with one known STI typically have a lifestyle that puts them at higher risk of becoming infected with HIV or other agents.

Chlamydia is the most commonly reported infectious disease in the United States and is caused by the obligate intracellular parasite *C. trachomatis*. There are 19 serovars in total. Serovars A, B, Ba, and C are isolated from trachoma, an eye infection that leads to blindness and is endemic in developing countries, mainly Africa. Serovars D to K are those associated with the genital tract disease chlamydia. Serovars L1, L2, L2a, L2b, and L3 are associated with **lymphogranuloma venereum (LGV)** (discussed later).

Rates of reported chlamydial infections have substantially increased since the mid-1990s. Public programs for screening and treatment of women were established to avert **pelvic inflammatory disease (PID)** and related complications. Several clinical trials evaluating the effectiveness of screening for the prevention of PID demonstrate that it is an effective intervention to reduce risk in young women. However, expansion of screening tests remains a priority. Chlamydia rates for women in 2018 were nearly two times higher than for men (692.7 vs. 380.6 per 100,000 persons). Worldwide, the rates of reported cases of chlamydia continue to increase, currently with about 127.2 million new cases reported annually.

In 2020, the CDC reported a total of 1,579,885 cases of genital *C. trachomatis* infection (Fig. 38.2). The national rate of reported chlamydia, 481.3 cases per 100,000 population, decreased by 13% compared with the rate in 2019. Although the rates of reported chlamydia decreased among both males and females in 2019–2020, the CDC supposed that the decreases were not probably due to decrease in new infections. Rather, during the COVID-19 pandemic, many health care clinics limited in-person visits to patients with symptoms or closed entirely. Because chlamydial infections are usually asymptomatic and are usually detected during annual reproductive health visits when STD screening are performed, such visits decreased in 2019–2020.

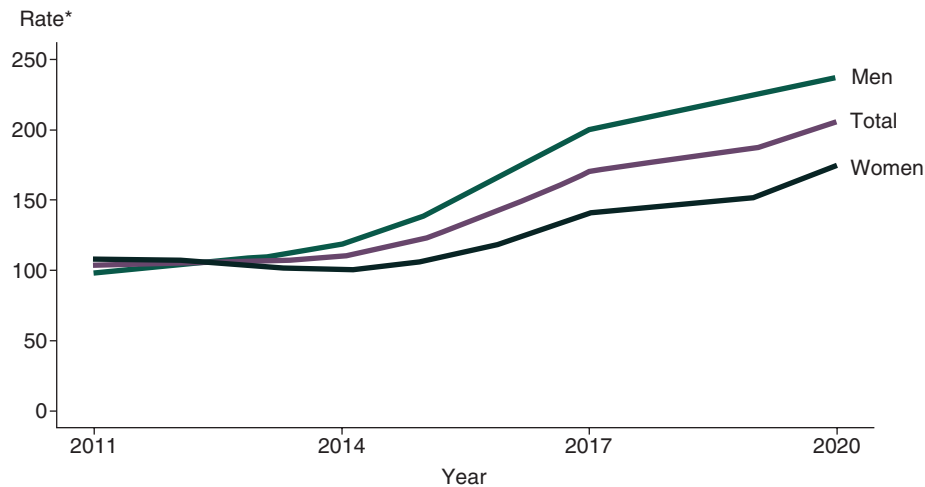


Fig. 38.1 Rates of reported cases of gonorrhea by sex 2011–2020. (From Centers for Disease Control and Prevention. (2020). *Sexually transmitted disease surveillance 2020*. Atlanta: Centers for Disease Control and Prevention.)

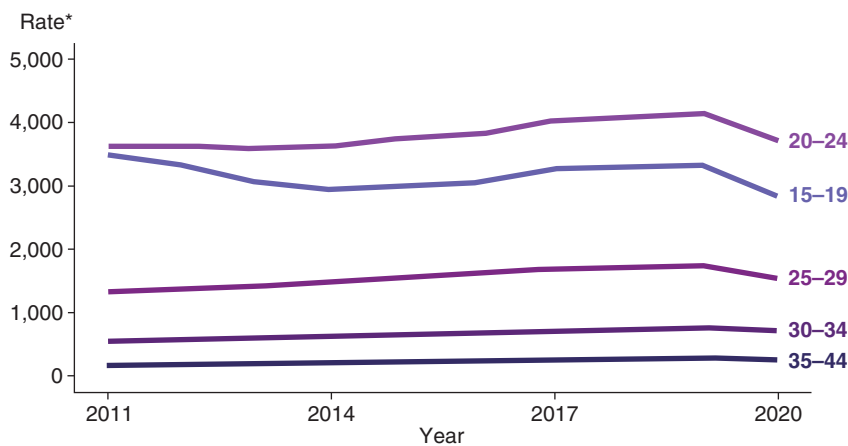


Fig. 38.2 Chlamydia: rates of reported cases by sex, United States, 2011–2020. (From Centers for Disease Control and Prevention. (2020). *Sexually transmitted disease surveillance 2020*. Atlanta: Centers for Disease Control and Prevention.)

Clinical manifestations

Gonococcal urethritis causes an acute infection with dysuria and urethral discharge usually without urinary frequency or urgency. *N. gonorrhoeae* binds specifically to columnar epithelial cells of the genitourinary tract, including the urethra, cervix, endocervix, and Bartholin glands. The organism also attaches to columnar epithelial cells of the anal canal, pharynx, and conjunctiva. From the time of exposure to the onset of symptoms, the incubation period averages 2 to 7 days, after which 90% or more of men experience a purulent urethral discharge, with the remaining 10% being asymptomatic (Fig. 38.3A). Infected women often have no symptoms for weeks or months, and the disease may be discovered only after being examined as a sexual contact of a male partner who tested positive for the disease.

The incubation period of NGU is 7 to 21 days, with symptoms arising less abruptly. Symptoms of urethritis in women include dysuria or pyuria and, less often, urethral discharge. Because urethritis in women is often accompanied by cervicitis (70% to 90%), a woman may also experience abnormal cervicovaginal discharge, intermenstrual bleeding, and pain

during or after vaginal sex. Patients with NGU are much more likely to be asymptomatic compared with patients with gonococcal urethritis. In symptomatic patients, NGU often results in a urethral discharge that is less profuse and more mucoid compared with that from gonococcal urethritis (see Fig. 38.3B). Dysuria without urethral discharge may be present in these patients.

If left untreated, spontaneous resolution occurs in most cases of urethritis in men; however, complications of ascending infection include acute prostatitis, **epididymitis** (a painful inflammation of the coiled tube [epididymis] at the back of the testicle), and urethral stricture. Ascending infection occurs in 10% to 20% of women with undiagnosed infections. These women are in danger of developing PID presenting as **salpingitis** (inflammation of the fallopian tubes), endometritis, internal abscesses, and chronic pelvic pain. Women with PID do not always have symptoms, but when symptoms are present, they can be very severe and can include lower abdominal pain, abnormal cervical discharge and bleeding, and fever. According to the American College of Gynecology, each year about 1,000,000 women in the United States develop acute PID, and more than 100,000



Fig. 38.3 **A**, Purulent urethral discharge in a patient with gonorrhea. **B**, Case of nonspecific urethritis with accompanying mild meatitis and mucoid urethral discharge. (A, From Marx. J., et al. [2010]. *Rosen's emergency medicine: concepts and clinical practice* (7th ed.). Philadelphia: Mosby; B, Courtesy Jim Pledger, Centers for Disease Control and Prevention, Public Health Image Library, Atlanta, GA.)

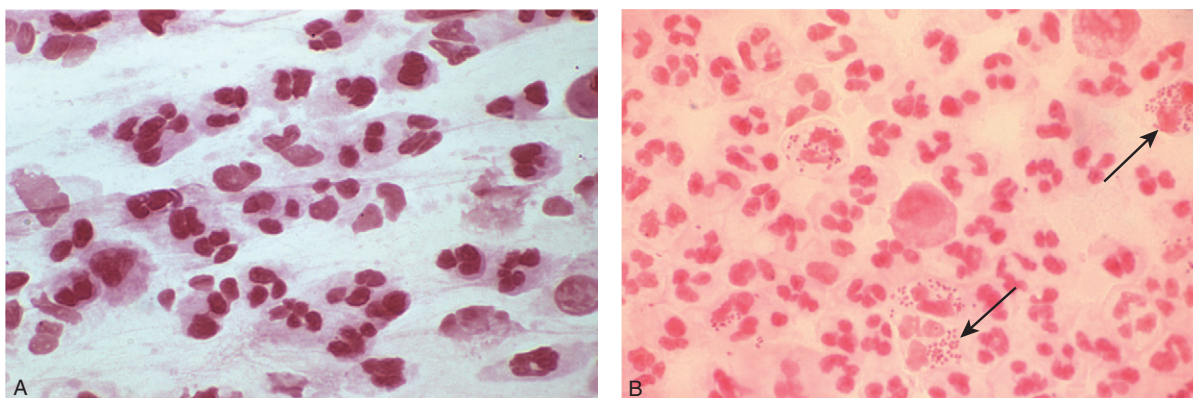


Fig. 38.4 **A**, Gram stain of a urethral exudate sample from a male with suspected urethritis ($\times 1000$). Numerous polymorphonuclear leukocytes are visible, confirming urethritis; however, no organisms are evident. This specimen proved to be negative for the presence of *Neisseria gonorrhoeae*. **B**, Gram stain of an acute case of gonococcal urethritis demonstrating gram-negative intracellular diplococci within leukocytes (arrows) ($\times 1000$). (A, Courtesy Dr. Norman Jacobs, Centers for Disease Control and Prevention, Public Health Image Library, Atlanta, GA; B, Courtesy Joe Miller, Centers for Disease Control and Prevention, Public Health Image Library, Atlanta, GA.)

of them will become infertile. In 0.5% to 3% of untreated cases of gonococcal urethritis, *N. gonorrhoeae* will invade the bloodstream and result in disseminated gonococcal infection. Perinatal exposure to *N. gonorrhoeae* and *C. trachomatis* can result in severe disease in newborns, including sepsis, pneumonia, inclusion conjunctivitis, and ophthalmia neonatorum, which can result in perforation of the globe of the eye and blindness.

Laboratory diagnosis

Urethritis can be diagnosed on the basis of the presence of mucopurulent or purulent urethral discharge; demonstration of two or more leukocytes per high-power field in a smear of urethral exudate; and a positive leukocyte esterase test result on first-morning voided urine or microscopic examination

of more than 10 WBCs per high-power field of first-morning voided urine sediment (Fig. 38.4A). Because of the potential for co-infection, the CDC recommends that all patients with confirmed or suspected urethritis be tested for gonorrhea, chlamydia, and HIV infection. Gram stain of male urethral exudate that demonstrates WBCs with gram-negative intracellular diplococci is considered a presumptive diagnosis (>99% specific and >95% sensitive) for *N. gonorrhoeae* in symptomatic men (see Fig. 38.4B). The sample is usually obtained by passing a small swab a few centimeters into the urethra or by milking any exudate onto a slide for staining. Because of the lower sensitivity, a negative Gram-stained smear result should not rule out gonococcal infection in asymptomatic males. If none of these criteria are present, the patient should be tested for *N. gonorrhoeae* and *C. trachomatis* and followed closely if test results are negative.

N. gonorrhoeae and *C. trachomatis* can be isolated in culture. *N. gonorrhoeae* is a fastidious organism that requires a selective growth medium (modified Thayer-Martin, Martin-Lewis, New York City media) in a carbon-dioxide (CO₂)-rich environment for culture. Cell culture using McCoy cells is considered the gold standard for chlamydia diagnosis. However, the testing method recommended for the most common organisms associated with urethritis is nucleic acid amplification tests (NAATs). NAATs are recommended for detection of *N. gonorrhoeae* and *C. trachomatis* because of their high sensitivity and specificity and the ease of specimen transport. There are several U.S. Food and Drug Administration (FDA)-approved NAATs available (Table 38.3).

Initially, approved specimens for testing via molecular assays included a first-morning voided urine sample or swabs from vaginal, endocervical, and urethral exudates. In May 2019, the FDA approved the Aptima Combo 2 Assay (Hologic, Marlborough, MA) and the Xpert CT/NG (Cepheid, Sunnyvale, CA) for extragenital diagnostic testing for gonorrhea and chlamydia using throat and rectal samples. The continued development of improved assays with increased testing percentages will hopefully result in earlier diagnosis and treatment for these patients.

Treatment

Treatment of patients presenting with urethritis should be initiated immediately to eradicate the organism. Originally, penicillin was the agent of choice for treating gonorrhea; however, penicillinase-producing *N. gonorrhoeae* strains developed. Fluoroquinolone agents, such as ciprofloxacin, replaced penicillin in the mid-1990s because of their greater efficacy, availability, and use as a single-dose, oral therapy. However, fluoroquinolone resistance began to increase rapidly by the early 2000s, becoming widespread in the United States and necessitating another change in treatment regimens. The

CDC currently recommends intramuscularly administered ceftriaxone for uncomplicated gonococcal infections.

Because many patients with gonorrhea may also be co-infected with *C. trachomatis*, the CDC recommends treatment with an antimicrobial regimen effective against both pathogens, such as ceftriaxone plus a single dose of azithromycin or doxycycline for 7 days. Alternative regimens include erythromycin, levofloxacin, or ofloxacin.

Recent reports indicate development of resistance against azithromycin and extended-spectrum cephalosporins. This is a cause of great concern because most therapeutic options for treatment may no longer be useful. In addition, with most cases of gonorrhea identified through molecular methods, there is no available culture to perform susceptibility testing. With few actual specimens submitted for culture, it becomes difficult to estimate accurately the antimicrobial resistance burden until after initial therapy fails in a patient. This portends a need to collect more specimens for culture and susceptibility testing and/or to identify and develop molecular tests for known antimicrobial resistance markers. In the meantime, patients should return for follow-up evaluation if symptoms continue despite therapy or recur after its completion. Sexual partners of these patients should be notified and treated. To avoid reinfection, partners are encouraged to abstain from sexual intercourse until therapy is completed.

Cervicitis

Causes

Cervicitis, an inflammation of the columnar and subepithelial cells of the endocervix, can be caused by infectious and noninfectious means. The infectious cases are more common and almost always associated with *C. trachomatis* and/or

Table 38.3 Molecular tests for gonorrhea and chlamydia approved by the U.S Food and Drug Administration

Test	Manufacturer	Test name	Method
<i>Chlamydia trachomatis</i>	BD Diagnostic Systems (Sparks, MD)	BD ProbeTec Q* CT amplified DNA assay	SDA
	Hologic/Gen-Probe (San Diego, CA)	Aptima CT assay CT assay	TC, TMA, DKA
<i>Neisseria gonorrhoeae</i>	BD Diagnostic Systems (Sparks, MD)	BD ProbeTec Q* GC amplified DNA assay	SDA
	Hologic/Gen-Probe (San Diego, CA)	Aptima GC assay competition assay (GC confirmation test)	TC, TMA, DKA
<i>Chlamydia trachomatis</i> , <i>Neisseria gonorrhoeae</i>	Abbott Molecular (Des Plaines, IL)	Abbott RealTime CT/NG	Real-time PCR
	BD Diagnostic Systems (Sparks, MD)	BD ProbeTec ET CT/GC amplified DNA assay	SDA
	Hologic/Gen-Probe (San Diego, CA)	Aptima COMBO 2 assay	TC, TMA, DKA
	Cepheid (Sunnyvale, CA)	Xpert CT/NG assay	Real-time PCR
	Roche Molecular Systems (Indianapolis, IN)	Cobas CT/NG test	Multiplex, real-time PCR

DKA, Dual kinetic assay; PCR, polymerase chain reaction; SDA, strand displacement amplification; TC, target capture; TMA, transcription-mediated amplification.

Modified from FDA-cleared/approved nucleic acid based tests, 2022. Available at: <https://www.fda.gov/medical-devices/in-vitro-diagnostics/nucleic-acid-based-tests>.

N. gonorrhoeae. Cervicitis can also result from trichomoniasis, genital herpes, or infections by *Mycoplasma* and *Ureaplasma*. Noninfectious inflammation occurs from injury to the cervix, usually as a result of a foreign object being inserted into the vagina, such as a birth control device, douche, or tampon. Chemical irritation can also occur because of the use of these products.

Epidemiology

Cervicitis is a very common condition, with more than 50% of all women developing it at some time in their adult lives. It is the most common STI in adolescent girls. Common risk factors for development of disease include beginning sexual activity at an early age, engaging in high-risk sexual behaviors, history of STIs, and having multiple sexual partners. It is considered the “silent partner” of male urethritis because symptoms are not always obvious, so many women may not feel the need to seek health care from a licensed provider. The lack of well-recognized symptoms and signs of cervicitis makes diagnosis very difficult. Patients in whom symptoms are not noted or recognized do not receive any treatment, and this leads to serious complications, such as PID, infertility, ectopic pregnancy, chronic pelvic pain, spontaneous abortion, and even cervical cancer.

Clinical manifestations

Symptoms of cervicitis, if present, may be fairly mild initially and progress over time. The most common symptom is a vaginal discharge that may be grayish or yellow, possibly with an odor. Other signs include abnormal bleeding, itching, irritation of the external genitalia, pain during intercourse, bleeding or spotting after intercourse, dysuria, and sometimes lower back or abdominal pain. Severe cases may demonstrate a profuse, almost puslike discharge accompanied by vaginal itchiness or abdominal pain.

Case check 38.2

Cervicitis is often difficult to diagnose because many of the symptoms may be mild or unrecognized. In some cases, symptoms may not be evident until much later, when potential complications, such as PID, chronic pelvic pain, ectopic pregnancy, or infertility, occur. By tracing all sexual contacts of an individual suspected of having an STI, it is hoped that screening and treatment can be performed before devastating sequelae occur. In the Case in Point, the patient's girlfriend did not complain of genital pain or dysuria, but she was also given antimicrobials in case she had an asymptomatic infection at the time.

Laboratory diagnosis

The diagnosis of cervicitis is generally performed clinically. However, microscopic analysis showing more than 10 WBCs per high-power field of exudate has been associated with chlamydial and gonococcal infection of the cervix. Whereas gram-negative intracellular diplococci from

urethral exudates are presumptive diagnostic of gonococcal infection in men, direct Gram-stained smear of cervical exudates is at most 50% accurate and is not commonly used. Most women presenting with symptoms of cervicitis are assessed for signs of PID and tested for *C. trachomatis* and *N. gonorrhoeae*. However, because the female genital tract is contiguous, there is some overlap between vulvovaginitis and cervicitis. Thus, if warranted, patients should also be evaluated for **bacterial vaginosis (BV)** and trichomoniasis.

Culture for any of these organisms lacks sensitivity compared with nucleic acid–based assays. The most common are the amplified tests, with very high rates of sensitivity and specificity (>95%). There are several FDA-approved assays available in automated and nonautomated formats (see Table 38.3). Rapid antigen tests are also available for point-of-care (POC) testing or physicians' office laboratories for chlamydia, *Neisseria*, trichomonads, and BV, but not for the Mycoplasmataceae. Although useful in the health care provider's office setting, the lack of sensitivity may require additional testing for confirmation of infection.

Treatment

Treatment options differ according to the organisms suspected or detected. Empiric therapy should be provided to patients with high-risk factors (age ≤ 25 years, new or multiple sexual partners, and unprotected sex), especially if there is a possibility that they will be lost to follow-up. As discussed previously, patients with *N. gonorrhoeae* and/or *C. trachomatis* are usually treated with multiple agents, including a cephalosporin and doxycycline or a macrolide, such as azithromycin or erythromycin, thus providing coverage for both organisms and lessening the chances of garnering resistance. Cases of trichomoniasis or BV are usually treated with metronidazole.

Women with persistent cervicitis should be evaluated for possible re-exposure to an STI or perhaps switched to a different antimicrobial regimen. Follow-up is recommended to ensure eradication of the organism(s). Sexual partners should also be notified and treated. The patient and partner(s) are encouraged to abstain from sexual intercourse to avoid reinfection until therapy is completed.

Vulvovaginitis

Causes

Vaginal infections account for more than 10 million visits to a health care provider annually. Vaginitis occurs when the mucosal lining of the vagina becomes inflamed and irritated. Bacteria, yeasts, or viruses are the main infectious causes, but chemical or mechanical irritants, such as feminine hygiene products, soaps, contraceptives, and some clothing, can also cause vaginitis. Typical signs include vaginal discharge, vulvar itching and irritation, and often odor. Three commonly associated diseases with these symptoms are BV, trichomoniasis, and candidiasis. The distinction among these diseases can initially be made by observations of the vaginal discharge.

Bacterial vaginosis

Epidemiology

BV is the most common vaginal infection in women of child-bearing age, although the role of sexual activity in women developing BV is still unclear. Prevalence in the United States is currently estimated to be 21.2 million (29.2%) among women 14 to 49 years of age based on a National Health and Nutrition Examination Survey (NHANES) report. Additional study findings noted that most women with BV (84%) were asymptomatic, and even though most sexually naïve women were rarely affected, they could also develop the infection. Most cases occur in sexually active women, with an increase in prevalence rates depending on the overall number of sexual partners. Risk factors for developing BV are dependent on a number of socio-demographic characteristics, such as initiating sexual activity at an early age; having numerous, anonymous, or frequently changed sexual partners; having female sexual partners; ethnicity (African Americans and Mexican Americans are at higher risk); and even frequent douching, which is thought to eliminate much of the normal protective lactobacilli.

Clinical manifestations

BV occurs when the delicate balance of the normal vaginal microbiota is disrupted and replaced by an overgrowth of specific organisms. In healthy women, the vaginal biota comprises mainly *Lactobacillus* spp., which maintain a pH between 3.8 and 4.5 to prevent the overgrowth and invasion of pathogenic bacteria by competitive exclusion, competition for nutrients, and release of antimicrobial substances, such as hydrogen peroxide, organic acids, bacteriocins, and biosurfactants. Thus, when vaginal lactobacilli are depleted, vaginal pH increases, allowing the overgrowth of various bacterial species, including *Gardnerella vaginalis*, *Mycoplasma hominis*, *Ureaplasma* spp., and anaerobes, such as *Prevotella*, *Porphyromonas*, *Peptostreptococcus*, and *Mobiluncus*. This mix of organisms leads to the development of an abnormal vaginal discharge, with a distinct unpleasant odor similar to that of rotting fish but lacks a true inflammatory reaction. Patients with BV demonstrate a discharge that is usually a milky, homogeneous, thin liquid adhering to the walls of the vagina. Discharge is often more apparent and odorous after intercourse (exposure to male ejaculate).

Laboratory diagnosis

BV can be diagnosed by using clinical criteria (i.e., Amsel diagnostic criteria) or Gram stain (considered the gold standard) of the vaginal discharge. The Amsel diagnostic criteria for BV require that three of the following four criteria be met: (1) clue cells on microscopic examination; (2) a thin, white, and homogeneous vaginal discharge; (3) a vaginal pH greater than 4.5; and (4) a fishy odor when 10% potassium hydroxide (KOH) solution is added to vaginal secretions ("whiff test"). Microscopic examination of the discharge is performed to look for the presence of clue cells, vaginal squamous epithelial cells typically coated with gram-variable *G. vaginalis*. Clue cells are identified with microscopic examination of a vaginal saline wet mount preparation or Gram stain. Normal vaginal squamous epithelial cells have distinct cell margins

and lack granularity, whereas clue cells tend to show sheets of coccobacillary organisms attached in clusters on the cell surface, making the border indistinct or stippled (Fig. 38.5). Polymorphonuclear leukocytes can also be demonstrated on the normal vaginal wet mount preparation but are rarely noted in cases of BV in the midst of clue cells.

Because of the lack of lactic acid-producing organisms to maintain an acidic environment, the vaginal pH begins to increase. This can be measured by using pH test paper to test the vaginal discharge and determine whether the vaginal environment is within the reference range. The amine or whiff test can also determine if the vaginal pH is higher than 4.5. This is performed by adding 1 or 2 drops of 10% KOH to the vaginal discharge and subsequently detecting a fishy odor. The odor is caused by volatilization of amines as a by-product of anaerobic metabolism. An alternative to the Amsel criteria is the use of direct Gram stain of vaginal secretions. Stained smears are read, and the numbers of morphotypes (*Lactobacillus*, *Gardnerella*, and *Mobiluncus*) present are scored using the Nugent scoring system (see Chapter 16).

Laboratory testing can also be performed to confirm BV by submitting a vaginal discharge specimen for culture. Identification of *G. vaginalis* is possible but usually presumptive, requires the use of specialized media, and is discouraged because of lack of specificity. The deoxyribonucleic acid (DNA) hybridization test Affirm VPIII (BD Diagnostic Systems, Sparks, MD) detects the most common causes of BV and vaginitis: *G. vaginalis*, *Candida* spp., and *T. vaginalis*. This test is easy to perform and can be completed in less than 1 hour but requires technical expertise and additional equipment. It definitively identifies the causative organism, thus providing focused therapy for the patient. Rapid card tests for use in a physician's office laboratory can detect proline aminopeptidase activity indicative of *G. vaginalis* in vaginal fluid specimens. Another POC option is the OSOM BV Blue test (Sekisui Diagnostics, Burlington, MA). It detects vaginal fluid sialidase, an enzyme associated with *G. vaginalis*, *Bacteroides*, *Prevotella*, and *Mobiluncus* with performance characteristics comparable to Gram stain.

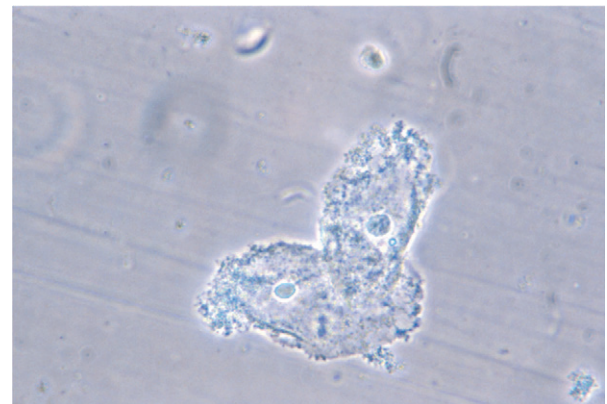


Fig. 38.5 Microscopic saline preparation of vaginal squamous epithelial cells with numerous bacteria (*Gardnerella vaginalis* attached; $\times 400$). Combined, these are identified as clue cells and are key indicators of bacterial vaginosis. (Courtesy M. Rein, Centers for Disease Control and Prevention, Public Health Image Library, Atlanta, GA.)

Treatment

BV is usually treated with an oral or a topical antimicrobial agent, such as metronidazole or clindamycin. Oral metronidazole therapy 500mg is a twice-daily, 7-day course, whereas metronidazole gel (0.75% one full applicator [5g]) or clindamycin cream (2% one full applicator [5g]) is applied intravaginally once a day for 5 and 7 days, respectively. The bacterial infection usually resolves within a few days, but a longer therapeutic period may become necessary if it recurs. Treatment is provided to relieve symptoms and reduce the risk of infectious complications with other organisms. BV in pregnancy can lead to potential adverse outcomes, such as preterm labor, low birth weight, premature rupture of membranes, and miscarriage. Thus pregnant women at high risk (previous preterm birth) should be managed carefully.

Case study

A 24-year-old female patient notes vaginal itching and irritation with a slight discharge. Previously, she had a yeast infection that had been successfully treated with over-the-counter (OTC) medications. Thinking that this was a recurrence, she self-treated again. This time, however, the symptoms did not resolve, and now there is a frothy discharge with a pungent odor. She presents to her health care provider for diagnosis, and the nurse practitioner takes a swab of the secretion to perform a rapid POC test and microscopy. A wet mount of the specimen demonstrates motile organisms.

Trichomoniasis

Epidemiology

Trichomoniasis, caused by the parasitic protozoan *Trichomonas vaginalis*, is the most common STI worldwide, according to the WHO, with approximately 156 million new cases among men and women 15 to 49 years of age reported each year. The greatest concentration of infections occurs in Southeast Asia and sub-Saharan Africa. It is a very common infection in the United States. The CDC estimates that 3.7 million people in the United States are infected with *T. vaginalis*, approximately 70% of whom show no signs or symptoms of infection. Although infections occur mostly in women, their male partners may also become infected. *T. vaginalis* infections are commonly associated with other STIs, in particular BV or gonorrhea. The number of cases of trichomoniasis is evenly distributed across all age groups and may be considered a useful marker for risky sexual behavior. Because this infection is easily cured and is not a reportable disease, it receives little emphasis from public health STI control programs and has a much lower priority compared with chlamydial and gonorrheal infections.

Clinical manifestations

Trichomoniasis demonstrates generalized symptoms of differing severity, ranging from mild irritation to severe

inflammation. Women typically report itching, burning, dysuria, lower abdominal pain, genital redness or soreness, and an odorous vaginal discharge that can be clear, white, yellowish, or greenish (Fig. 38.6). The vaginal discharge also has an elevated pH, between 5.0 and 6.0. The cervix demonstrates a punctate and papilliform appearance, which gives the appearance of a strawberry, hence the term *strawberry cervix* (Fig. 38.7). This feature is best detected by colposcopy and rarely during routine examination.

The high rate of asymptomatic infections makes screening very important in reducing overall infection. The severity of symptoms depends on a multitude of factors that influence



Fig. 38.6 Yellowish-green frothy purulent discharge from the cervical os, demonstrative of trichomoniasis. (Courtesy Centers for Disease Control and Prevention, Public Health Image Library, Atlanta, GA.)

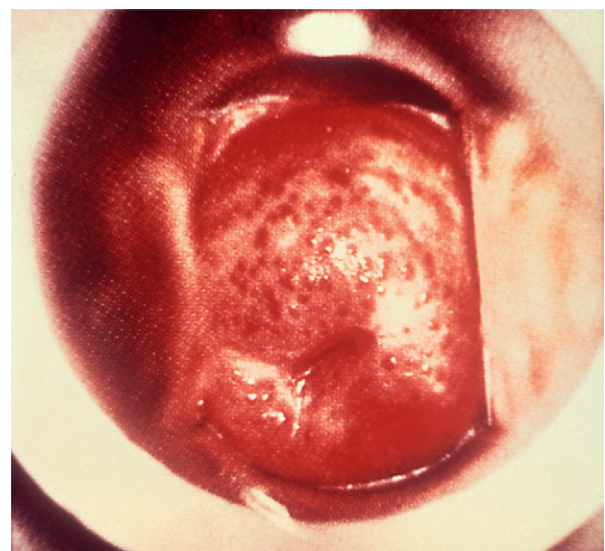


Fig. 38.7 Presentation of a strawberry cervix caused by a *Trichomonas vaginalis* infection. The cervical mucosa reveals punctate hemorrhages along with accompanying vesicles or papules. (Courtesy Centers for Disease Control and Prevention, Public Health Image Library, Atlanta, GA.)

the host inflammatory response, such as hormonal levels, coexisting vaginal biota, and the strain and relative concentration of the organisms present in the vagina. Additionally, some of the virulence factors of this organism are adherence factors, which enable colonization of cervicovaginal epithelial cells, and cysteine proteases, which degrade host extracellular matrix proteins.

Male sexual partners can also be infected with *T. vaginalis*, presenting with symptoms of NGU. A number of complications are associated with infection by *T. vaginalis*. Cellulitis has been seen in some patients following hysterectomy. A significant correlation has been found between infection and low birth weight, premature rupture of membranes, and preterm delivery. African studies also confirm increased potential for acquisition of HIV infection with *T. vaginalis* infection.

Laboratory diagnosis

Trichomoniasis can be easily determined through visualization of motile trichomonads in a saline preparation of vaginal fluid (Fig. 38.8). Physicians may perform this test in the clinic because organisms lose viability quickly after collection of the sample. This is allowable under Clinical Laboratory Improvement Amendments (CLIA) rules as provider-performed microscopy. Despite the ease of the test, it has limited sensitivity, ranging from 60% to 70%. Culture is still considered the gold standard, which requires Diamond medium, although this is not widely available. A commercially available IPouch TV self-contained culture media device (Biomed Diagnostics, White City, OR) has been demonstrated to be as good as culture and has been used successfully with clinician-obtained and self-obtained specimens. A delayed inoculation technique is also possible, allowing initial reading of the wet mount preparation and then inoculation of the culture pouch if the wet mount preparation is negative. Swab specimens may sit at room temperature for up to 30 minutes before pouch inoculation.

Newer commercially available methods for diagnosis include an office-based oligonucleotide probe test (Affirm VPIII, BD Diagnostic Systems) and a POC rapid antigen detection test (OSOM *Trichomonas* rapid test, Sekisui Diagnostics). The approximate sensitivity of these assays ranges from 71.4% to 83 compared with wet mount. These tests may be of

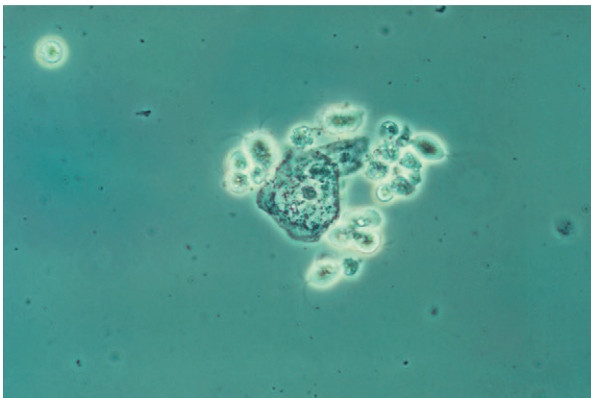


Fig. 38.8 Phase contrast wet mount micrograph of vaginal discharge revealing the presence of *Trichomonas vaginalis* protozoa surrounding a squamous epithelial cell ($\times 200$). (Courtesy Centers for Disease Control and Prevention, Public Health Image Library, Atlanta, GA.)

value in settings in which microscopy is not possible. Several high-throughput nucleic acid amplification platforms have been FDA approved. These include the BD Probe Tec TC Q^x Amplified DNA assay (BD Diagnostic Systems), Aptima TV assay (Hologic), Xpert TV assay (Cepheid), and AmpliVue *Trichomonas* assay (Quidel, San Diego CA).

Treatment

Metronidazole is the drug of choice for treating trichomoniasis. Approximately 4% to 10% of all cases display some level of resistance to treatment with metronidazole, although this can usually be overcome with higher oral doses. Patients who are allergic to metronidazole may be treated with tinidazole, which actually has a longer half-life for a shorter therapeutic duration. All sexual partners should be treated, along with patients with asymptomatic infection. Otherwise, if left untreated, they may later become symptomatic, and transmission of infection will continue.

Candidiasis

Epidemiology

Vulvovaginal candidiasis is symptomatic vaginitis caused by infection with the yeast *Candida*. Almost all cases of infection are caused by *C. albicans*, with only a small percentage caused by other species of *Candida* (*C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. krusei*). Yeast infections are also termed *vaginal thrush* or *moniliasis*. Infection is very common, with almost 75% of all adult women having had at least one genital yeast infection in their lifetime. *Candida* is always present in the body in small amounts, so when an imbalance occurs, such as changes in the acidity of the vagina, symptoms manifest. Vaginal candidiasis is not considered a true STI, but it is included in this discussion because it causes symptoms in the female genital tract that are similar to the organisms described earlier. Rarely, candidal infections can be passed sexually to male partners, but these infections are usually not serious and respond well to treatment.

Yeast infections are the most common genital infections affecting women. Infections occur as a result of changes in the vaginal biota. In many cases, infection may follow a course of broad-spectrum antimicrobials that were administered for another purpose but inadvertently eliminated the normal biota. Hormonal causes of yeast infections include pregnancy, ovulation, menopause, oral contraceptives, and estrogen replacement therapy. Immunosuppressive conditions, such as diabetes mellitus, iron deficiency, or HIV infection, will also lead to an increased infection rate. Finally, various skin conditions (psoriasis, lichen planus, or lichen sclerosus) can lead to infection. The highest prevalence of infections occurs typically in women of childbearing age who are producing ample amounts of estrogen, which causes the lining of the vagina to mature and contain glycogen, a preferred growth substrate for *C. albicans*.

Clinical manifestations

Women with vulvovaginal candidiasis usually experience vulvar itching, burning, pain, swelling, or redness with an abnormal vaginal discharge ranging from a slightly watery, white discharge to a thick, white, cottage cheese

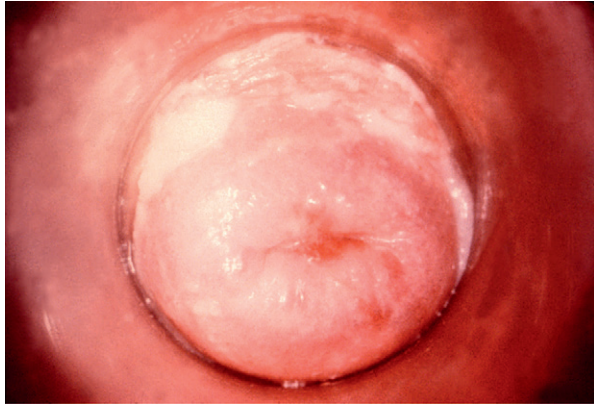


Fig. 38.9 Candidal cervicitis caused by *Candida* spp. The cervical discharge is white, thick, and curdlike. (Courtesy Centers for Disease Control and Prevention, Public Health Image Library, Atlanta, GA.)

curd-like discharge (Fig. 38.9). Additional signs and symptoms include vulvar inflammation and redness, sometimes spreading widely in the groin to include the pubic area, inguinal area, and thighs; pain during intercourse; and painful urination. Males who develop genital candidiasis demonstrate an itchy rash or burning at the foreskin or head of the penis called **balanitis**.

Laboratory diagnosis

The diagnosis of yeast infections is typically done clinically. Discharge can be submitted for microscopy and culture to rule out other causative agents of vaginosis. Microscopic evaluation of a yeast infection demonstrates budding yeast forms on Gram-stained smears and KOH preparation. The pH of the vaginal fluid remains unchanged as opposed to an elevated pH with BV and trichomoniasis.

Treatment

Yeast infections are typically treated with OTC medications or prescription antifungal agents. In most cases, symptoms usually disappear completely with adequate treatment. However, recurrences are possible that may signal some other underlying health issues, such as immunosuppression. Numerous antifungal drugs, both prescription and OTC medications, are available to treat yeast infections, but all are essentially azole antifungal agents that inhibit the production of ergosterol, which is an important component of the fungal plasma membrane. Such agents are available as suppositories, topical creams or lotions, and oral pills. The agent used may depend on whether the infection is self-diagnosed or diagnosed by a health care provider. The duration of therapy is agent specific and can correspond to as little as a single dose or treatment lasting up to 7 days. Women with first-time infections should seek advice from their provider as should those who seem to have a chronic or recurrent form of disease (four or more proven episodes). Women with a weakened immune system may require a longer therapeutic regimen and should also consult their provider before resorting to self-treatment.

Despite therapy, chronic or recurrent infections may still occur. This may be from inadequate initial treatment, or perhaps a secondary infection may develop as a result of prolonged scratching, causing the skin of the vulva to become cracked and raw. In many cases, women will opt to self-treat as opposed to visiting their health care provider to save time and money. However, unless women are fully aware of the signs and symptoms of candidiasis, their diagnosis may be incorrect. A high percentage of all OTC drugs sold to treat candidiasis were probably used by women without the disease, thus increasing the drug resistance rate. It is now thought that women who experience recurrent yeast infections have persistent infection and not reinfection.

Methods of prevention suggested to reduce the incidence rate of yeast infections include taking antimicrobials as prescribed and managing diabetes by keeping blood glucose levels under control. Other methods include wearing cotton undergarments and loose-fitting clothing to avoid persistent and excessive moisture in the genital area. Women should avoid wearing wet bathing suits or exercise clothing for long periods. Douching can kill bacteria that help control the growth of *Candida* and is therefore not recommended. Prescription oral medications may be taken regularly on the recommendation of the health care provider. Treatment of the sexual partner is not recommended unless balanitis is present.

Case check 38.3

Vulvovaginitis is one of the most common reasons why females seek medical attention or treatment. In many women, all three causes of disease (i.e., non-lactic acid-producing bacteria, *Candida* spp., or *Trichomonas*) can initially produce asymptomatic infections. In the Case in Point, the girlfriend is at a higher risk of developing one or more of these forms throughout her lifetime because of her young age, especially if she has had multiple sexual partners and participates in unprotected sex (no barrier, e.g., condom).

Genital ulcer disease

Causes

Genital ulcer disease is characterized by a disruption of the skin and/or mucosal layer of the genital region. In the United States, most patients who present with genital ulcers have herpes, syphilis, or chancroid. Less common causes of genital ulcers include LGV and donovanosis. The frequency of each infection differs by geographic area and patient population; however, genital herpes is the most prevalent of these diseases. Multiple infections may be present in a patient with genital ulcers. Agent-specific diagnosis based solely on clinical evaluation of the ulcers is often confounded by the overlapping patterns of clinical presentation and occurrence of multiple and mixed infections. It is very important to successfully treat genital ulcer disease because studies have demonstrated higher susceptibility to multiple STIs, especially an increased risk of HIV susceptibility and transmission.

Case study

A 14-day-old female infant is brought to her pediatrician with a 2-week history of eye discharge. The infant was delivered vaginally at full term. Following delivery, the infant continued to squint, only opening her eyes in the dark. She was referred to an ophthalmologist, who noted large, bilateral corneal ulcers. These ulcers were scraped and submitted for culture, which grew HSV type 2. When the patient's mother was questioned about any illnesses during pregnancy, she mentioned a scabby labial rash and dyspareunia (abnormal pain during sexual intercourse caused by a spasm) during the pregnancy, which resolved after about 1 week. Serologic testing of the mother was also positive for HSV.

Genital herpes

Epidemiology

Genital herpes is a chronic viral infection caused by HSV. There are two serotypes of HSV, HSV-1 and HSV-2, both of which can cause genital herpes, but HSV-2 is more commonly detected (about 90% of the time). Although HSV-2 typically affects the genital area via sexual contact, HSV-1 is mainly associated with an oral infection of the lips, gums, tongue, and other areas inside the mouth, commonly referred to as *cold sores* or *fever blisters*, occurring primarily through orofacial contact. Both serotypes produce mucosal ulcers that are clinically indistinguishable.

Reports from a variety of clinical settings reveal that genital herpes is the most common patient request for treatment of symptomatic genital ulcers in the United States. According to the NHANES and the National Disease and Therapeutic Index (NDTI), the prevalence of HSV-2 infection increased dramatically from 1976 to 1991 and again from 2002 to 2009. There was a slight increase during 2011 to 2012, however recent studies suggest that the upward trend of HSV-2 seroprevalence has been reversed. HSV infections are not nationally reportable diseases, so determination of new infections is based on initial office visits in office practices reporting to the NDTI. The CDC estimated the prevalence of HSV-2 in 2018 at over 18 million, with approximately 1.3 million infections in the 15- to 24-year age group. Genital HSV-2 infection is more common in women than in men (15.9% vs. 8.2%). This disparity is most likely because male-to-female transmission is more prevalent than female-to-male transmission. From 70% to 90% of infected individuals are unaware of their infections because they are asymptomatic or have only vague symptoms. Many patients with subclinical disease have episodes of viral shedding from anogenital sites and represent a significant reservoir of transmission.

Clinical manifestations

HSV infection of oral or genital tissue is initiated by mucosal contact with an active (primary or recurrent) infection of another individual. The virus enters the body through direct sexual contact of skin or mucous membranes with the secretions or mucosal surfaces of an infected person. Incubation periods range from 2 to 14 days after initial exposure. Most individuals with genital herpes are asymptomatic or show minimal signs or symptoms and have not received a

diagnosis of genital herpes. Despite a lack of symptoms, these patients continue to shed HSV into the genital tract intermittently. Therefore the disease is often transmitted by those who are unaware that they are infected or who are asymptomatic when transmission occurs.

In symptomatic patients, primary infection with HSV-2 manifests with extensive, painful vesicles, fever, myalgia, malaise, dysuria, and inguinal lymphadenopathy. These systemic effects are usually limited to the primary episode. About 10% of primary genital HSV-2 infections also result in herpes meningitis, which usually resolves without complications within 1 week. Primary lesions last 2 to 6 weeks as vesicles and then become ulcerous and contain large quantities of infectious HSV particles (Fig. 38.10). In primary infections, viral shedding can last 15 to 16 days, with new lesions continuing to form for up to 10 days after exposure. Asymptomatic viral shedding can continue for up to 1 year after resolution of the primary clinical presentation, increasing the risk of transmission to sexual partners. Lesions of primary genital herpes typically ulcerate and heal inward from the periphery without scarring within 3 weeks. Ulcers that form on dry surfaces (buttocks or thighs) heal more rapidly than those on mucocutaneous surfaces (vagina, cervix, glans of the penis). Women tend to have more severe disease caused by genital herpes infection, including constitutional symptoms and complications, compared with men. This disparity may be the result of the larger affected surface area of the female genital tract and the ability of HSV to spread more easily over the abundant moist surfaces.

After recovery from primary infection, HSV ascends along the sensory nerve roots to the dorsal root ganglion and establishes latency. Reactivation of the virus occurs on exposure to stressors, including extensive exposure to sunlight, menstruation, malnutrition, fatigue, anxiety, and subsequent viral infections. With reactivation, the virus travels from the dorsal root ganglion back down the nerve root to cause recurrent outbreaks of symptoms. Although clinical presentation is indistinguishable, recurrent disease caused by HSV-1 generally recurs less regularly compared with genital infections with HSV-2. Recurrent disease typically results in milder symptoms compared with those of the primary infection, and the lesions heal more quickly. Approximately 90% of patients with genital herpes have at least one recurrence during the first year after primary infection. The median recurrence rate for genital HSV-2 is four episodes per year, with an average of 50 days to first recurrence. Over time, the frequency of recurrence lessens.

The most serious complication of genital herpes is neonatal transmission. Neonatal infection with HSV usually occurs during delivery (85%) but can also occur congenitally (5%) or postnatally (10%). Symptoms of neonatal HSV disease acquired during delivery usually appear in the first 9 to 11 days of life. Exposure to vaginal secretions containing HSV can result in a variety of clinical manifestations, including localized disease of skin, eyes, and mucosa; central nervous system (CNS) involvement; or disseminated disease. The mortality rate for neonates with CNS or disseminated disease can be as high as 85%. Risk of transmission from mother to child during delivery is high (30% to 50%) among women who acquire the disease during the third trimester and is low (<1%) in women with a history of recurrent herpes at

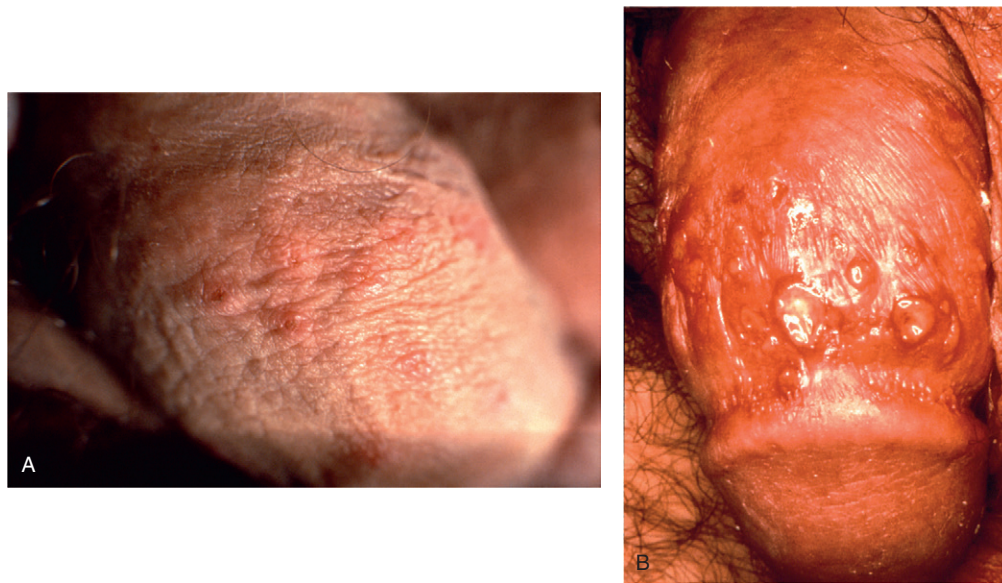


Fig. 38.10 **A**, Vesicles of herpetic lesions on the penile shaft caused by herpes simplex virus 2. **B**, Maculopapular herpetic rash on the penile shaft and corona of the glans penis. After the vesicles break, tender ulcers may remain that might take 2 to 4 weeks to heal the first time they appear. (A, Courtesy Susan Lindsley, Centers for Disease Control and Prevention, Public Health Image Library, Atlanta, GA; B, Courtesy Dr. N. J. Flumara and Dr. Gavin Hart, Centers for Disease Control and Prevention, Public Health Image Library, Atlanta, GA.)

term or who acquire genital HSV infection during the first half of pregnancy. It is thought that maternal antibodies from recurrent infections protect the neonate from serious disease. Other serious complications of genital HSV infection include corneal infection and fatal sporadic encephalitis in immunocompromised hosts.

Laboratory diagnosis

Viral isolation was the most common method for the diagnosis of HSV infections. The best specimens for culture are vesicle fluids collected with a syringe or swab. Cervical swabs and mucosal surface swabs are also acceptable specimens. Specimens for culture from mucocutaneous lesions must be taken as early as possible in the disease process. The likelihood that a herpetic lesion will produce a positive culture diminishes with each day after the appearance of the lesion. Specimens should be placed in viral transport medium, shipped cold, and not frozen. If crusting lesions are the only possible specimen for culture, vigorously collected specimens containing epithelial cells from the lesion help increase the sensitivity of culture. Viral samples can be stored at 4° C; however, specimens being kept for longer periods should be stored at –70° C.

HSV grows rapidly on numerous cell culture lines, and the cytopathic effect (CPE) in culture is usually seen within the first 24 hours in most cases, but some isolates can take as long as 14 days to develop a CPE. The most common cell lines used for viral culture include mink lung, rhabdomyosarcoma, MRC-5, HEp-2, and A549 cells. Infected cells develop intracellular granulations, enlarge, and then display intracellular inclusions (Fig. 38.11). Viral culture is a reliable method to detect HSV because it is highly specific and relatively inexpensive. However, the sensitivity ranges from 30% to 95%, depending on the stage of the lesion and whether it

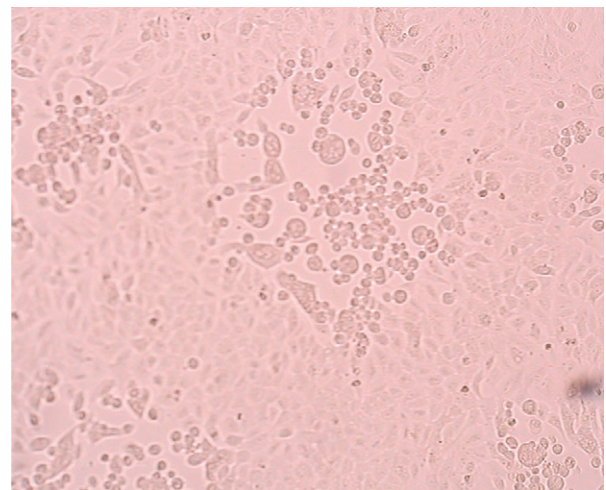


Fig. 38.11 Culture results of a genital specimen inoculated into A549 cells. Cytopathic effects demonstrate rounded, swollen, refractile cells (unstained, $\times 100$). (Courtesy Bonnie Hill, Brooke Army Medical Center, San Antonio, TX.)

is a primary infection or recurrence. The sensitivity of viral culture is especially low for recurrent lesions.

Because of the low sensitivity of viral culture and because other viruses can mimic the CPE of HSV, confirmation using monoclonal antibodies against type-specific epitopes is recommended. The CDC recommends serotyping viral culture isolates to determine whether infection is caused by HSV-1 or HSV-2. Other available confirmatory tests include genetically engineered cell lines, EIAs, the enzyme-linked virus-inducible system (ELVIS; Diagnostics Hybrids, Athens, OH).

NAATs have generally replaced cell culture as the diagnostic method of choice because of their sensitivity, specificity,

and rapid results. NAATs are regarded as the most sensitive methods for direct detection of HSV. Depending on the gene targets, an assay can detect HSV-1 and HSV-2 or distinguish between HSV-1 and HSV-2. Examples include the MultiCode-RTx kit (Luminex, Austin, TX), ProbeTec HSV-Q^x test (BD Diagnostic Systems), and AmpliVue HSV 1 + 2 assay (Quidel, San Diego, CA).

Treatment

Genital herpes is a chronic, life-long viral infection for which there is no cure. Antiviral chemotherapy is widely used to treat mucocutaneous and genital herpes, offering some clinical benefits to most symptomatic patients. Clinical trials have demonstrated that acyclovir, valacyclovir, and famciclovir are effective antivirals for the treatment of genital herpes. These drugs can reduce the signs and symptoms of genital herpes when used to treat primary infection and recurrent episodes. No treatment currently exists that will eradicate latent virus or reduce the risk, frequency, or severity of recurrences after therapy is discontinued. Suppressive therapy can reduce the frequency of recurrences by 70% to 80% in patients who have more than six recurrences per year.

The safety and efficacy of suppressive therapy have been demonstrated in patients receiving daily therapy with acyclovir for up to 6 years and with valacyclovir or famciclovir for 1 year. Not only does daily therapy decrease the frequency and duration of recurrences, but it also limits viral shedding, reducing disease transmission rates. Clinicians also use suppressive therapy to prevent transmission of HSV from mother to child during delivery. Neonates exposed to HSV infection during delivery should be closely monitored for symptoms of disease. Treatment with intravenous (IV) antiviral chemotherapy can reduce the overall morbidity and mortality of neonatal herpes. The CDC recommends IV administration of acyclovir for treatment of neonatal herpes (20mg/kg body weight IV) every 8 hours for 21 days for disseminated and CNS disease or for 14 days for disease limited to skin and mucous membranes.

Case study

A 23-year-old homeless female patient addicted to crack cocaine is arrested during a drug raid and brought to jail. During her in-processing physical examination, she admits to routinely exchanging sex for drugs to maintain her drug habit. The health care provider notes a rash on the prisoner's palms and soles. Samples of the lesions are obtained, and spirochetes are identified through dark-field microscopy. A serum sample is also taken and submitted to the laboratory for rapid plasma reagin (RPR) screening and confirmational tests for syphilis.

Syphilis

Epidemiology

Syphilis is a genital ulcer disease caused by the spirochete *Treponema pallidum* subsp. *pallidum* (*T. pallidum*). *T. pallidum* is an obligate parasite of humans, with no other known animal hosts or environmental reservoirs. Despite the existence of effective prevention measures, such as barrier prophylaxis, and treatment options, syphilis remains a global problem, with an estimated 12 million people infected each year. The incidence of syphilis in the United States immediately following World War II was close to 500,000 cases per year. The number of reported infections decreased by 89.7% during the 1990s, and in 2000, the rate was the lowest since reporting began. Primary and secondary syphilis rates increased from 2001 to 2009, and the CDC reported 13,997 infections in 2009. In 2010, the overall rate of new syphilis infections decreased for the first time in 10 years, with 13,774 cases (4.5 cases per 100,000 people). However, the number of cases increased every year from 2011 to 2020 (Fig. 38.12). In 2020, 41,655 cases of primary and secondary syphilis and 133,945 cases of all stages of syphilis were reported in the United States. The rate of primary and secondary syphilis infections has increased almost every year since the historic low in 2000, increasing 6.8% during 2019–2020.

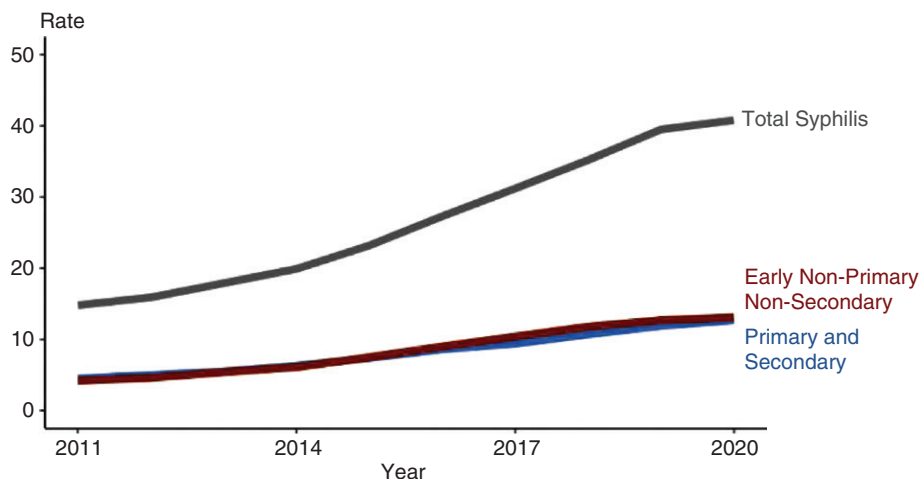


Fig. 38.12 Rates of reported cases of syphilis by stages of infection. (Modified from Centers for Disease Control and Prevention. [2020]. Sexually transmitted disease surveillance, 2020. Atlanta: Centers for Disease Control and Prevention.)

Not surprisingly, the incidence of congenital syphilis mimics the rate for primary and secondary syphilis. The average yearly rate of congenital syphilis decreased by 14.1% from 1996 to 2005, representing a total 74.2% decrease overall during that period. Unfortunately, the rate of congenital syphilis increased from 2006 to 2008, but it decreased to 334 cases in 2012. From 2013 to 2020, there was a steady increase, with 2148 cases reported in 2020.

Most reportable cases of syphilis occur in the western United States and in urban areas in other parts of the country. The CDC estimated that the proportion of primary and secondary syphilis cases attributable to MSM increased by 58% from 2000 to 2004. Beginning in 2005, the CDC required all state health departments to report the sex of partners of persons with syphilis. Data from the 44 states and the District of Columbia that provided information about the sex of sexual partners indicate that the highest percentage (53.5%) of primary and secondary syphilis cases in 2018 occurred in MSM. Most of these new infections are occurring in the young African American gay community.

Clinical manifestations

Syphilis is a multistage disease, with diverse and wide-ranging clinical manifestations. *T. pallidum* is an invasive organism that can enter the host through any site and initiate infection. There are four stages in the pathogenesis of syphilis: primary, secondary, latent (hidden), and tertiary syphilis. The incubation period from initial exposure to development of the primary **chancre** ranges from 10 to 90 days. Infection occurs when *T. pallidum* penetrates dermal microabrasions or intact mucous membranes, typically producing a single, painless, nonsuppurative lesion, called a *chancre*, at the site of inoculation (Fig. 38.13). Appearance of the initial chancre, usually in the anogenital region, signals primary syphilis. Moderate regional lymphadenopathy is associated with the primary stage. The chancre usually becomes indurated and will progress to ulceration but typically is not purulent. In some cases, syphilis occurs without a visible ulcer. The chancre has an indurated raised edge and is extremely infectious. The lesion will spontaneously heal in 3 to 6 weeks (but this can be as long as 12 weeks) if the patient does not receive antimicrobial therapy. The disappearance of the chancre does not signal an end to the infection.



Fig. 38.13 Chancres on the penile shaft resulting from a primary syphilitic infection caused by *Treponema pallidum*. Painless lesions are indurated with raised edges. (Courtesy M. Rein, Centers for Disease Control and Prevention, Public Health Image Library, Atlanta, GA.)

Treponemes gain access to the bloodstream in untreated patients, and this spirochetemia is responsible for the signs and symptoms of secondary syphilis, which begin about 6 weeks to 6 months after infection. Clinical manifestations of secondary syphilis include, but are not limited to, rash, mucocutaneous lesions, and lymphadenopathy (Fig. 38.14). This is when the organisms are at their highest numbers. The hematogenous spread of the treponemes causes fever, lymphadenopathy, myalgia, and anorexia. Almost any organ can be involved in secondary syphilis. Signs of organ system involvement include hepatitis, osteitis, and keratitis. CNS involvement can also occur during the secondary phase. Most patients develop macular or papular skin lesions involving the trunk, soles of the feet, and palms. Fluid from these so-called *nickel-and-dime lesions* is extremely infectious. Another result of secondary syphilis is the appearance of **condylomata lata**, which are mucoid, wartlike growths (Fig. 38.15). These lesions



Fig. 38.14 Close-up view of keratotic lesions on the palms of a patient's hands caused by a secondary syphilitic infection. Each lesion is full of treponemes. (Courtesy Robert Sumpter, Centers for Disease Control and Prevention, Public Health Image Library, Atlanta, GA.)

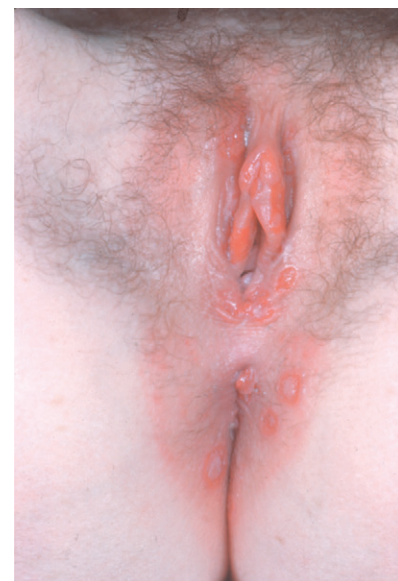


Fig. 38.15 Close-up view of condylomata lata lesions involving the vulva and anal region caused by a secondary syphilitic infection. Each lesion is full of treponemes. (Courtesy Centers for Disease Control and Prevention, Public Health Image Library, Atlanta, GA.)

often occur in the perianal region but can occur in other moist regions of the body. Even without treatment, the symptoms of secondary syphilis disappear within a few weeks of onset, and the patient enters the latent phase. Patients are no longer infectious after secondary syphilis lesions heal; however, relapses occur in about 25% of untreated patients during the first year after infection.

Latent syphilis is divided into two stages: early latent and late latent syphilis. Patients are considered to have early latent syphilis during the first year after infection, whereas late latent syphilis is defined as asymptomatic infection lasting longer than 1 year or with an unknown duration. The serologic testing result during the late latent stage is positive, and the patient is asymptomatic. Sexual transmission of disease is unlikely during the latent period, but organisms may enter the bloodstream intermittently and can infect the developing fetus during pregnancy. Latent syphilis is characterized by cerebrospinal fluid (CSF) abnormalities in the absence of symptoms. Indicators include elevated protein levels, depressed glucose levels, and lymphocytic pleocytosis (the presence of WBCs in CSF).

Many untreated patients relapse during the first year of the latent period and will suffer a recurrence of the symptoms associated with secondary syphilis. Other patients may never relapse, and a third group of patients will experience tertiary syphilis. Tertiary syphilis is associated with immune sequelae of primary and secondary syphilis, rather than the direct presence of treponemes. These patients may develop neurologic symptoms, cardiovascular effects (e.g., aneurysm), or late benign syphilis. In the latter condition, the patient develops nonspecific granulomatous lesions, called **gummas** (Fig. 38.16). Gummas can occur in skin, bone, or viscera and cause a localized form of tissue and bone destruction. The lesions rarely resolve without appropriate antimicrobial therapy. The late stages of syphilis develop in about 15% of untreated patients and can occur decades after they initially acquired the disease.

T. pallidum can cross the placenta and cause congenital infection in neonates. Up to 40% of all infants infected while in the womb are stillborn or die shortly after birth. Therefore effective detection and prevention rely on routine serologic screening during the first prenatal visit. In areas in which risk

for congenital syphilis is high, additional serologic screening and obtaining a sexual history should occur at 28 weeks' gestation and on delivery. If the mother's test results indicate syphilis, antimicrobial agents are administered to treat her and the fetus. Infection across the placenta is most likely to occur during the primary or secondary stages of syphilis, but the fetus can be infected at any time during pregnancy.

Congenital syphilis is divided into two stages: early disease and late disease. At birth, neonates with early congenital syphilis usually develop symptoms 2 to 10 weeks after delivery and show signs of secondary syphilis, including failure to thrive, fever, collapse of the nasal bridge (saddle nose), watery nasal discharge, hepatosplenomegaly, jaundice, rash, condylomata lata, meningitis, and periostitis. Untreated children can go on to develop late congenital syphilis. These patients are asymptomatic at birth but can develop blindness, deafness, arthritis, bone and joint pain, deformed lower leg bones (saber shins), and abnormal notched and peg-shaped teeth (Hutchinson teeth) as young children.

Laboratory diagnosis

T. pallidum does not survive outside a mammalian host, and all attempts to propagate the organism continuously *in vitro* have been unsuccessful. Dark-field microscopy and DFA for *T. pallidum* (DFA-TP) examination of patient specimens are considered the definitive methods for diagnosing early syphilis from lesion exudates. Today, blood tests are more commonly performed. Antibodies produced in response to infection with *T. pallidum* are detectable during primary syphilis, and their levels continue to increase as the disease progresses to the secondary stage.

Serologic tests for the diagnosis of syphilis are divided into two general types: **nontreponemal antibody tests** and **treponemal antibody tests**. The nontreponemal antibody tests, such as the rapid plasma reagin (RPR) test, unheated serum reagin assay, toluidine red unheated serum test (TRUST), and Venereal Disease Research Laboratory (VDRL) test are used as screening tests and detect the presence of antibodies to cardiolipin and other lipoidal indicators of tissue damage. These tests are not highly specific but usually correlate with disease activity. Febrile infections, pregnancy, and autoimmune disorders may produce biologically false-positive reactions with nontreponemal tests. A fourfold increase in titer between acute-phase and convalescent-phase sera is necessary to demonstrate a clinically significant difference between two nontreponemal test results obtained by the same methods. Clinicians can use quantitative nonspecific tests to follow the efficacy of treatment because these antibody levels decrease and ultimately disappear with the successful treatment of syphilis.

Patients with reactive nontreponemal test results are then tested with assays using specific treponemal antigens to confirm the diagnosis. These confirmatory tests are specific treponemal antibody tests and include the EIAs, fluorescent treponemal antibody absorption (FTA-ABS), chemiluminescence immunoassays (CIAs), immunoblot, and *T. pallidum* particle agglutination (TP-PA) tests. The most common tests performed in the United States are the TP-PA test and EIAs. These help distinguish true- and false-positive nontreponemal test results and the diagnosis of late latent or tertiary syphilis. Treponemal antibody tests cannot be used to follow therapy because these antibodies do not disappear following the successful treatment of



Fig. 38.16 Gumma found on the nose of a patient with tertiary syphilitic *Treponema pallidum* infection. Without treatment, a chronic infection will begin to damage the internal organs, including the brain, nerves, eyes, heart, blood vessels, liver, bones, and joints. (Courtesy J. Pledger, Centers for Disease Control and Prevention, Public Health Image Library, Atlanta, GA.)

the disease. Most patients remain seropositive for life, regardless of the treatment or its success; however, 15% to 25% of patients who received treatment early in the disease revert to being serologically nonreactive within 2 to 3 years.

The CDC reported on the results of reverse screening for syphilis by several large reference laboratories. Because of high test volume and availability of automatable treponemal EIAs and CIAs, these facilities were performing the treponemal antibody test as the screening assay and following up all reactive test results by using the nontreponemal antibody test as the confirmatory assay. Interestingly, it was noted that 56.7% of all reactive treponemal antibody results were non-reactive by the RPR test. Because of this notable discordance, the CDC recommended performing an additional, different treponemal antibody test (TP-PA) when the results from the first two tests do not match.

The diagnosis of congenital syphilis is complicated by the ability of maternal nontreponemal and treponemal IgG antibodies to cross the placenta. This passive transfer of antibodies makes serologic test interpretation in neonates difficult. To differentiate maternal antibody from the infant antibody to syphilis, an IgM-specific FTA-ABS test may be useful. Current testing recommendations suggest that infants born to mothers who have reactive serologic test results should be evaluated with a quantitative nontreponemal serologic test (RPR or VDRL) performed on infant serum. Confirmation of congenital syphilis includes clinician suspicion based on symptoms and history along with demonstration of treponemes in body tissues or fluids.

Treatment

The CDC recommends a single intramuscular dose of benzathine penicillin G for the treatment of primary and secondary syphilis. Penicillin therapy has been the mainstay of treatment for over 50 years because *T. pallidum* isolates appear to be uniformly susceptible to β -lactam drugs. Current guidelines suggest multiple doses of aqueous or procaine penicillin G for suspected cases of neurosyphilis. A single dose of penicillin G is not as effective as multiple doses because low levels of the drug are maintained in CSF. Ceftriaxone, amoxicillin, and ampicillin are also effective for the treatment of early syphilis.

For patients allergic to penicillin, the CDC recommends doxycycline (100 mg orally twice daily for 14 days) or tetracycline (500 mg four times daily for 14 days) as an alternative regimen. Tetracycline and doxycycline are not recommended during pregnancy because of cosmetic concerns regarding staining of fetal primary dentition. Pregnant patients who are allergic to penicillin should be desensitized and treated with penicillin. The use of macrolides, such as azithromycin and erythromycin, for the treatment of syphilis is not recommended because of reported resistance.

Chancroid

Epidemiology

Chancroid, or soft chancre, is an STI caused by the fastidious, gram-negative bacterium *Haemophilus ducreyi*. Chancroid is a major cause of genital ulcerative disease in Africa, Southeast Asia, the Caribbean, and Latin America, and it is of increasing concern in the United States because of its role as a potential

cofactor in the transmission of HIV. Because it is difficult to confirm *Haemophilus ducreyi* infections microbiologically, the global epidemiology of chancroid is poorly documented, although UNAIDS and the WHO estimate that the annual global incidence of chancroid is approximately 6 million cases. Studies from regions in which chancroid is endemic revealed that most cases of the disease occurred in patients with direct exposure to commercial sex workers.

The disease was endemic throughout many parts of the world well into the 20th century. The prevalence of chancroid decreased steadily in the developed world before the discovery of antimicrobial agents, largely because of a reduction in mass migrations and tighter regulation of the commercial sex industry. According to the CDC, reported cases of chancroid decreased steadily from 1987 to 2001, when 38 cases were reported in the United States. Since 2002, the number of cases has fluctuated, with a decreasing trend. Only three cases of chancroid were reported in 2018. This trend most likely reflects a decrease in the incidence of this disease; however, rates of infection may be underreported because *H. ducreyi* is very difficult to culture. Many patients with chancroid are also co-infected with *T. pallidum* or HSV.

Clinical manifestations

Chancroid is characterized by painful genital ulceration, inflammatory inguinal adenopathy, and bubo formation. The pathogen enters through microabrasions in skin after sexual intercourse. Human inoculation experiments demonstrated that a dose of one colony-forming unit will result in infection at 50% of inoculation sites. A tender erythematous papule develops at the site of infection 4 to 7 days after exposure before progressing to the pustular stage. Once the papule develops into a pustule, it will usually rupture 2 to 3 days later to form painful, shallow ulcers with granulomatous bases and purulent exudates. The chancroid usually develops on the external genitalia of men and women. The sites of infection in men include the prepuce, coronal sulcus, glans, and shaft; in women, the labia, clitoris, vaginal wall, and cervix can be involved. At first glance, chancroid ulcers appear similar to syphilitic chancres and herpetic lesions but can be differentiated. The chancroid is a painful lesion with ragged or uneven, soft edges and a gray to yellow purulent exudate compared with the painless chancre, which has a hard indurated edge, and the painful herpetic lesion, which starts out with vesicles that later burst to reveal clear fluid and develop yellow crusts. The chancroid ulcer may persist for weeks or months, with slow resolution in the absence of antimicrobial therapy.

Painful, tender inguinal lymphadenitis occurs in almost 50% of all patients with chancroid. Lymphadenopathy in these patients is usually unilateral and is more frequently observed in men. If left untreated, swollen lymph nodes can develop into fluctuant buboes (Fig. 38.17), which can rupture spontaneously if not drained. Extragenital manifestations of chancroid are rare, but lesions have been observed on the inner thighs, breasts, and fingers.

Laboratory diagnosis

Clinical evaluation and laboratory culture of *H. ducreyi* is considered the gold standard for the diagnosis of chancroid. Ulcer lesion material and/or bubo aspirate may be submitted



Fig. 38.17 Early chancroid lesions on the penis and groin, along with accompanying regional lymphadenopathy. Chancroid lesions are painful and have ragged or uneven edges. (Courtesy Dr. Pirozzi, Centers for Disease Control and Prevention, Public Health Image Library, Atlanta, GA.)

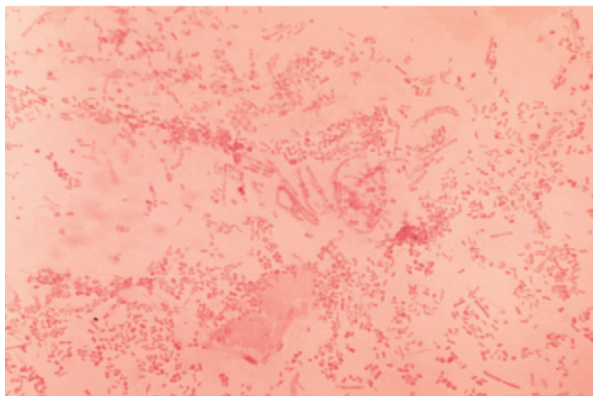


Fig. 38.18 Direct Gram-stained smear microscopic examination reveals the presence of gram-negative coccobacilli indicative of a chancroid infection (×1000). (Courtesy Joe Miller and Dr. N.J. Fiumara, Centers for Disease Control and Prevention, Public Health Image Library, Atlanta, GA.)

for direct smear examination and culture. Microscopically, *H. ducreyi* is a pleomorphic, gram-negative coccobacillus that occasionally occurs in characteristic chains or so-called *school of fish* formations (Fig. 38.18). However, Gram-stained smears alone are not sensitive or specific for the diagnosis of chancroid because the specimen often includes numerous bacterial contaminants.

H. ducreyi is extremely fastidious and requires nutritious media for growth. Some clinical isolates grow well on chocolate agar, but enriched media can increase culture sensitivity. Two commercially available varieties of enhanced media contain GC agar base, hemoglobin, IsoVitalax, and fetal calf serum, and another has Mueller-Hinton agar with chocolate horse blood and IsoVitalax. Most *H. ducreyi* strains grow well at 33° to 35° C in a 5% CO₂ atmosphere. It is critical that specimens be sent as quickly as possible to the laboratory for successful culture. Even with the optimal combination of

media and timely delivery to the laboratory, culture is only about 80% sensitive.

Testing methods that are alternatives to culture, such as PCR and immunoassays, are being developed to increase the sensitivity of chancroid diagnosis. Currently, no FDA-approved PCR assays are commercially available in the United States. Multiplex PCR assays specific for the causative agents of genital ulcer disease have been privately developed and are performed in a few laboratories that have completed validation studies. Preliminary studies indicate that these assays are more rapid and sensitive than culture. Serologic detection of chancroid has been demonstrated by EIAs that contain antibodies against *H. ducreyi* lipooligosaccharide and antigen detection using DFA, but these assays are not widely available and have cross-reactivity problems. Given the limitations of alternative testing methods, culture remains the primary diagnostic test performed by most microbiology laboratories for suspected cases of chancroid.

Treatment

Antimicrobial therapy for the treatment of patients with chancroid resolves the clinical symptoms, prevents transmission, and cures the disease. The CDC's 2015 sexually transmitted diseases treatment guidelines include a single oral dose of azithromycin, orally administered ciprofloxacin (twice daily for 3 days), orally administered erythromycin (three times daily for 7 days), or a single intramuscular dose of ceftriaxone. Patients with underlying immunosuppression or HIV infection and uncircumcised male patients should be carefully treated. Genital ulcers heal more slowly in these patients, and persistence of *H. ducreyi* in the lesions has been documented. In uncircumcised male patients, lesions under the foreskin also take longer to heal. Given the difficulty of culturing *H. ducreyi* and the limitation of sensitive testing methods, presumptive treatment for chancroid should be considered if HSV infection and syphilis are ruled out. Chancroid should also be considered if the patient lives in or has traveled to an endemic area or to an area with an ongoing outbreak.

Lymphogranuloma venereum

Epidemiology

LGV is an STI caused by serovars L1, L2, L2a, L2b, and L3 of the intracellular bacterium *C. trachomatis*. Compared with serovars A to K, LGV strains are more virulent in experimental animal models of infection and more invasive in humans. LGV rarely occurs in the United States and other industrialized countries. Most LGV infections occur in Africa, Southeast Asia, Central and South America, and the Caribbean countries. The prevalence of the disease is unclear because of difficulty in clearly distinguishing it from other causes of genital ulceration and bubo formation, such as chancroid. The actual number of cases in the United States is unknown. However, the CDC reported a cluster of cases among MSM occurring between August 2015 and April 2016 in Michigan.

Clinical manifestations

C. trachomatis serovars predominantly infect monocytes and macrophages, passing through the epithelial surface to regional lymph nodes and causing disseminated infection.

Clinical LGV has three stages: primary, secondary, and tertiary. The primary lesion produced by *C. trachomatis* usually presents as a small genital or rectal lesion, which ulcerates at the site of transmission. It is possible for a primary lesion to be absent, or it may simply go unnoticed in the urethra, vagina, or rectum. Symptoms typically occur after a 3- to 30-day incubation period following exposure. **Proctitis** (rectal inflammation) can occur as a primary manifestation of infection following direct inoculation of the rectal mucosa, and symptoms may arise within days of exposure.

The secondary stage of LGV involves the spread of *C. trachomatis* from the initial site of inoculation via the lymphatics to regional lymph nodes. This stage begins 2 to 6 weeks after the development of the initial lesion and primarily affects the inguinal lymph nodes. The most commonly noted manifestation is a very painful unilateral inguinal and/or femoral lymphadenopathy. Pressure from the inguinal ligament separating the groin lymph nodes gives rise to the groove sign, once believed to be diagnostic for LGV, but occurring in only 20% of cases (Fig. 38.19). Affected nodes can coalesce to form buboes, which may then ulcerate and discharge pus from multiple points.

The inguinal form of LGV is more common in men because lymphatic drainage from the penis is to the inguinal lymph nodes. In women, lymphatic drainage of the vagina and cervix is to the retroperitoneal lymph nodes. As a result of enlargement and suppuration of the perirectal and pelvic lymphatics, LGV commonly presents as backache in women, with initial lesions on the cervix and upper vagina, or presents in the rectal area in MSM who engage in receptive anal intercourse. Rectal involvement presents as an acute ulcerative proctitis with bloody purulent rectal discharge, pain, tenesmus, and constipation and may be misdiagnosed as ulcerative colitis.



Fig. 38.19 Presentation of lymphogranuloma venereum with bilateral inguinal lymphadenopathy that discharges purulent material (buboes). The inguinal ligament separates inguinal and femoral nodes to create the so-called groove sign. (From Habif, T. [2009]. *Clinical dermatology: a color guide to diagnosis and therapy* [5th ed.]. London: Mosby.)

Systemic symptoms, such as fever, malaise, and headache, can occur during the secondary stage of LGV. Infection with *C. trachomatis* (all serovars) is not restricted to the genital region. Submaxillary and cervical gland lymphadenitis can result from exposure to *C. trachomatis* organisms during oral sex. Ocular inoculation causes conjunctivitis along with preauricular lymphadenopathy.

In the tertiary stage of the disease, untreated infections can lead to chronic inflammation and destruction of affected tissues. Patients with untreated LGV proctitis may develop rectal damage, strictures, and, in women, rectovaginal fistulae. If LGV remains untreated, lesions caused by *C. trachomatis* often lead to fibrosis and granulomas. Fibrosis can lead to lymphatic obstruction, causing elephantiasis of the genitalia in men and women and esthiomene in women that results in a destructive, hypertrophic, granulomatous enlargement of the vulva and subsequent ulceration. Tertiary-stage sequelae do not typically disappear once antimicrobial therapy is initiated, and surgical intervention is required to correct many late complications, such as rectal stricture.

Laboratory diagnosis

The diagnosis of LGV is based on clinical presentation, epidemiologic information, and the exclusion of other causes of genital or rectal ulcers, inguinal lymphadenopathy, and/or proctitis. Isolation of *C. trachomatis* from specimens requires cell culture using HeLa-229 or McCoy cell lines. Cell culture used to be considered the gold standard, but the technique is no longer widely used because it is difficult to perform and has a low sensitivity.

Because of low sensitivity, DFA and EIA tests are not recommended for the direct detection of *Chlamydia*. Therefore NAATs have become the preferred diagnostic method for detecting genital *C. trachomatis* infections because of their high sensitivity and specificity. Many research articles have noted that self-collected specimens are almost as sensitive as health care provider-collected specimens. Depending on the manufacturer, urine specimens also yield similar sensitivities, thereby eliminating the more invasive specimen collection process.

Molecular identification is a two-step procedure. The first step confirms the presence of *C. trachomatis*. Available methods include PCR, strand displacement amplification, and transcription-mediated amplification. These assays are approved for the diagnosis of genital infections, and research shows high sensitivity and specificity in rectal infections, but they are not FDA-approved for rectal chlamydial infections. Specimens containing *C. trachomatis* advance to the second step for serovar determination by real-time or real-time quadruplex PCR assays. Detection of *C. trachomatis* in bubo or lymph node material by any of these diagnostic methods is highly suggestive of LGV, especially within an endemic area; however, detection of the organism in ulcer material can support the diagnosis only if the strain is identified by sequencing or typing assays.

Three serologic techniques for *C. trachomatis* infection are available: complement fixation, microimmunofluorescence, and EIA. Complement fixation and microimmunofluorescence titers greater than 1:64 along with high anti-MOMP antibodies indicating a positive result support LGV diagnosis in an appropriate clinical context. LGV serologic

test interpretation is not standardized, and *C. trachomatis* serovar-specific serologic tests are not widely available. Serology has a limited use in the diagnosis of *Chlamydia* infections.

Treatment

Current CDC treatment guidelines for LGV include the use of orally administered doxycycline twice daily for 21 days, or erythromycin orally four times a day for 21 days. A course of therapy lasting at least 3 weeks is recommended because of the deep tissue penetration by the organism. Doxycycline is not recommended for use in pregnant or lactating women. An alternative treatment recommended for these patients is erythromycin, four times daily for 21 days. Azithromycin may prove useful for treatment of LGV in pregnancy, but minimal clinical data are available on its effectiveness for the treatment of LGV. Anorectal LGV has an excellent prognosis if the disease is diagnosed and treated early. Delays in treatment can lead to serious complications, such as abscesses, fistulae, and rectal strictures. In settings without access to specific diagnostic LGV testing, the CDC recommends treating patients who present with symptoms consistent with LGV, such as proctitis, genital ulcer disease, or inguinal lymphadenopathy.

Patients with both LGV and HIV infection should receive the same treatment regimen as that for HIV-negative individuals. Prolonged antimicrobial therapy may be required for HIV-positive patients, and symptoms may resolve more slowly. Sexual contacts of patients in whom LGV has been diagnosed should be notified within 60 days of symptom onset to be evaluated and treated. Prompt treatment can prevent symptom development and lessens the risk of the original patient becoming reinfected. Sexual intercourse should be avoided until the course of treatment is completed and symptoms have disappeared completely.

Donovanosis

Epidemiology

Donovanosis, also known as **granuloma inguinale**, is a genital ulcerative disease caused by the intracellular, gram-negative bacterium *Klebsiella granulomatis* (formerly *Calymmatobacterium granulomatis*). The disease is rare in the United States but is endemic in some tropical and developing areas, including India, Papua New Guinea, central Australia, Brazil, and southern Africa. Ulcers are most common in uncircumcised men with poor standards of genital hygiene. Disseminated donovanosis may spread to bone and liver and is often associated with complications during pregnancy and cervical infection. Cases in the United States typically involve someone who is from or has recently traveled to an endemic region.

Clinical manifestations

Donovanosis is thought to be spread by direct contact with lesions during sexual activity; however, nonsexual transmission has been documented. Donovanosis often produces a firm papule or subcutaneous nodule that ulcerates at the primary site of inoculation. Ulcerative lesions usually develop in the genital region but may occur at oral, anal, or

other extragenital sites. Lesions frequently appear in warm, moist surfaces, such as the folds between the thighs, perianal region, scrotum, or labia. Local lymphadenopathy occurs, leading to ulcerative lesions developing in the skin overlying the infected lymph nodes. Four types of donovanosis have been described in the literature:

1. Ulcerogranulomatous—the most common type, with painless, beefy red ulcers that bleed to the touch and are well-defined, friable, nontender, and nonindurated with profuse granulation tissue (Fig. 38.20)
2. Hypertrophic (verrucous)—usually with a raised, irregular edge and sometimes completely dry
3. Necrotic—offensive-smelling ulcer causing tissue destruction; common in patients with long-standing donovanosis
4. Sclerotic or cicatricial—with fibrous or scar tissue

In men, donovanosis frequently affects the penis, scrotum, glans penis, and/or anus. In women, the disease primarily affects the labia minora, fourchette, and, less often, the cervix and upper genital tract. Pregnant women have a higher risk for developing disseminated donovanosis, with spread to bone and liver predominating. Extragenital exposure may result in ulceration of the lips, cheeks, and gums. If left untreated, the disease process may result in extensive destruction of the genitalia and spread to distant sites by autoinoculation. It is considered to be only mildly contagious; repeated exposure may be necessary for clinical infection to occur.

Laboratory diagnosis

Diagnosis of donovanosis is usually determined clinically based on a detailed history and physical examination findings of ulcerative lesions in a patient with sexual contact in an endemic region. Diagnosis can be confirmed by



Fig. 38.20 Progressively ulcerative, highly vascular, beefy red lesions caused by *Klebsiella granulomatis* (donovanosis). (Courtesy Joe Miller and Dr. T. Tabua, Centers for Disease Control and Prevention, Public Health Image Library, Atlanta, GA.)

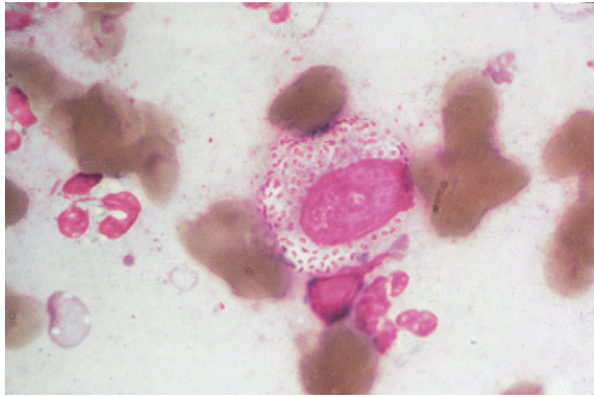


Fig. 38.21 Wright-stained smear of biopsy sample of granuloma inguinale lesion revealing Donovan bodies, encapsulated rods of *Klebsiella granulomatis* within macrophages ($\times 1000$). (From Rakel, R. E., & Rakel, D. P. (2011). *Textbook of family medicine* [8th ed.]. Philadelphia: Saunders.)

performing Giemsa or Wright stains of tissue and identifying characteristic intracellular **Donovan bodies** within macrophages (Fig. 38.21). Smears obtained directly from tissue or biopsy samples can be examined. Donovan bodies measure 0.5 to 0.7 mm $\times$ 1 to 1.5 mm in diameter and may or may not be encapsulated.

K. granulomatis is an extremely fastidious organism and cannot be grown on conventional bacteriologic media. It will only grow in eggs, peripheral blood mononuclear cells, and a few cell lines, so culture is beyond the capability of most clinical laboratories. PCR assays have been developed to detect *K. granulomatis*, but no FDA-approved test is commercially available. A DFA assay was developed using antigen derived from donovanosis lesions. These assays have low sensitivity for detecting early infection and are not widely available.

Treatment

Very few studies on donovanosis treatment have been reported in the literature. Effective treatment halts the progression of destructive ulcers, but therapy should continue until granulation and reepithelialization of the lesions occur. The CDC recommends azithromycin, taken orally once a week for 3 weeks, until all symptoms resolve. Alternative antimicrobials include doxycycline, ciprofloxacin, erythromycin, and trimethoprim-sulfamethoxazole. Doxycycline and ciprofloxacin are contraindicated in pregnant women. Pregnant and lactating women should be treated with erythromycin or azithromycin. Individuals with both donovanosis and HIV should receive the same treatment regimen as those who are HIV negative. Addition of an aminoglycoside should be considered to increase the chances of successful resolution. Patients should be followed up clinically until symptoms of the disease have resolved. Recurrence of symptoms can occur 6 to 18 months after symptom resolution, even after apparently effective therapy was completed. Sexual contacts within the 60 days preceding the onset of symptoms should be identified and examined for disease. Once the lesions have healed, extensive deformity and functional disability may require surgical repair.

Case check 38.4

In the United States, the three most common genital ulcer diseases are herpes, syphilis, and chancroid, with herpes being the most common. Infected individuals develop a latent infection, so recurrences may appear later. In the Case in Point, there is no mention of a genital ulcer disease, but in some cases, ulcers are not readily visible and may not be sought if there is no pain. This also stresses the need to obtain a detailed medical history and to ask appropriate questions, including about individual customs, to get a full health assessment for the patient.

Acquired immunodeficiency syndrome

Case study

A 21-year-old gay male patient presents to the local STD clinic with complaints of tenesmus, rectal discomfort, bloody stools, and a purulent discharge. There are also a few nonpainful ulcerative lesions noted around the anus. The patient's social history indicates that he has had multiple partners since a breakup with his boyfriend more than 6 months ago. He routinely chats with potential partners online to arrange anonymous encounters. He reports commonly participating in receptive anal intercourse and anilingus. His health care provider swabs the ulcers, takes a second swab from the rectum, and orders blood work for HIV screening. The following laboratory test results are discussed with the patient: molecular assay for *C. trachomatis* and *N. gonorrhoeae* was positive for *N. gonorrhoeae*; RPR test was positive; titration of 1:32, FTA-ABS test was positive; and HIV1/2 antigen/antibody immunoassay was negative. The patient received a diagnosis of concurrent gonorrhea and syphilis.

Causes

Human immunodeficiency virus (HIV) is a lentivirus in the family Retroviridae and is the causative agent of AIDS. It is a non-icosahedral, enveloped virus with two copies of a single-stranded RNA genome. HIV mainly targets CD4⁺ lymphocytes, which become depleted over time, leaving the body susceptible to many opportunistic infections because of the impaired immune system (Fig. 38.22). Individuals become infected with HIV through various routes of viral exposure—cervicovaginal, penile, rectal, oral, percutaneous, IV, or in utero.

To infect a cell, viral surface glycoproteins bind to receptors on the CD4⁺ lymphocyte, which allows the viral envelope to fuse with the cell plasma membrane. The viral contents are released into the cell, where the RNA genome is reverse-transcribed into DNA, transported to the cell's nucleus, and integrated into the host cell DNA as a provirus. The HIV provirus produces HIV proteins and enzymes to form new viral particles. These HIV particles are released by budding from the cell to infect other cells throughout the body.

The viral genome encodes a number of structural proteins (gag, pol, and env), regulatory proteins (tat and rev), and accessory proteins (vpu, vpr, vif, and nef). Structural proteins are necessary for viral replication because they are associated

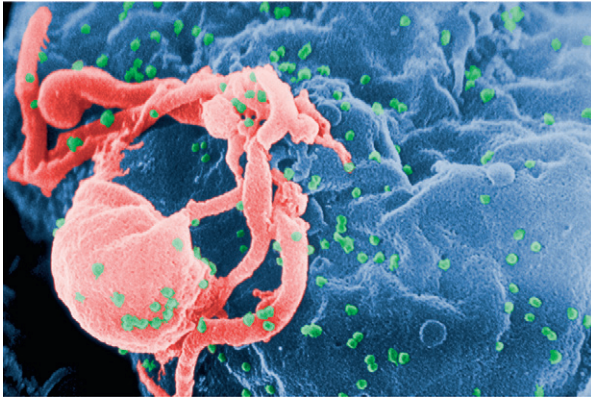


Fig. 38.22 Colorized scanning electron micrograph of human immunodeficiency virus 1 virions (green) budding from a cultured lymphocyte (pink) ($\times 20,000$). (Courtesy C. Goldsmith, P. Feorino, E. L. Palmer, W. R. McManus, Centers for Disease Control and Prevention, Public Health Image Library, Atlanta, GA.)

with the viral core (gag), polymerase (pol), and the viral envelope (env). Also, the three enzymes necessary for multiplication include a reverse transcriptase to transcribe viral RNA into DNA, an endonuclease that enables the DNA to be integrated into the host cell, and a protease that cleaves proteins to help viral maturation.

Epidemiology

Currently, there are two known types of HIV: HIV-1 and HIV-2. HIV-1 is the predominant virus worldwide, with estimates of almost 7000 persons becoming infected daily. HIV-2 is less pathogenic and geographically limited primarily to West Africa and European immigrants from West Africa. The AIDS pandemic began in the early 1980s, and in the beginning, the overwhelming majority of reported cases occurred in the male homosexual population. Other potential risk factors at that time included IV drug use, being of Haitian origin, and having hemophilia A and receiving blood transfusions. Federal health officials feared that tens of thousands of homosexual men had the acquired immune dysfunction, which they likened to an immunologic time bomb.

Initially, there was a rapid annual increase in the number of reported HIV infections and AIDS-related deaths. In the early years, the stigma of a positive test result was daunting; with few treatment options available, death was inevitable. To date, almost 33 million people worldwide have died from AIDS-related causes. When combination **highly active antiretroviral therapy (HAART)** gained widespread use, the number of deaths decreased, especially in developed countries. In 2019, there were 690,000 AIDS-related deaths, representing a 60% decrease since 2004. People infected with HIV are now surviving longer, thus increasing the overall number of people with HIV.

As of 2019, the WHO estimated that there are 1.65 million people newly infected and 38.0 million individuals worldwide with HIV. Approximately two thirds of these infected individuals reside in sub-Saharan Africa. Other areas of high infection prevalence are noted in several states from the former Soviet Union. In higher-income countries, such as the United States, Britain, and those in western Europe, the total

number of people with HIV continues to increase, largely because of access to better health care and HAART.

According to the CDC, approximately 1.2 million individuals currently have HIV infection in the United States, of whom approximately 14% are unaware that they are infected. Male homosexuals still develop most of the infections annually, especially in the African American population. However, in today's society, almost one third of all new HIV infections occur in heterosexuals engaging in risky behaviors. In 2018, African Americans accounted for approximately 42% of all new infections. Several outreach and educational awareness programs have been developed to promote frequent screening of individuals, especially those who are considered to be at high risk for infection (IV drug use, exchanging sex for drugs, unprotected sex, sex with multiple or anonymous partners, previous STIs). Although the percentage of infected and unaware persons is decreasing, screening and educational efforts must continue because these individuals are most likely to continue to spread the disease to further contacts.

Clinical manifestations

HIV infection can be considered a progressive disease noted by three specific phases: acute infection, clinically latent infection, and immunodeficiency (AIDS). HIV infection leads to gradual deterioration of the immune system over time as the virus continues to replicate and infect more cells, gradually decreasing the number of CD4⁺ T lymphocytes. The loss of these T cells leads to overwhelming opportunistic infections caused by numerous viruses (cytomegalovirus, varicella-zoster virus, HSV, and JC virus), bacteria (*Mycobacterium* and *Salmonella*), fungi (*Candida*, *Coccidioides*, *Histoplasma*, *Cryptococcus*, and *Pneumocystis*), and protozoa (*Cryptosporidia*, *Cystoisospora*, and *Toxoplasma*) that affect almost every organ system.

Certain serious and life-threatening diseases are termed *AIDS-defining illnesses* (Box 38.1), indicating that the patient has reached the advanced stage of HIV infection, that is, AIDS. As a public health measure for surveillance and reporting, HIV infection and disease have been defined and classified into five stages, 0 to 5 (Table 38.4). The current CDC classification system categorizes infection by increasing severity, all levels of which require laboratory-confirmed evidence of HIV infection in addition to the CD4⁺ T lymphocyte count or CD4⁺ percentage of total lymphocytes.

Acute infection

The acute primary HIV-1 infection is very difficult to recognize and diagnose because of the transient nature of the illness. Symptoms are noted in 40% to 80% of patients within 2 to 4 weeks after primary infection and resolve within 7 to 14 days. The symptoms overall are fairly nonspecific, resembling a mononucleosis-like syndrome. Individual symptoms commonly noted include fever, maculopapular rash, oral ulcers, lymphadenopathy, malaise, weight loss, arthralgia, pharyngitis, and night sweats. During this acute phase, plasma viral particle levels reach high levels of up to 100 million copies of HIV-1 RNA per milliliter of plasma. Additionally, viral particles are destroying CD4⁺ T lymphocytes and seeding lymphoid organs and other tissue reservoirs for widespread dissemination.

BOX 38.1 AIDS-defining conditions

- Bacterial infections, multiple or recurrent^a
- Candidiasis of bronchi, trachea, or lungs
- Candidiasis of esophagus^b
- Cervical cancer, invasive^c
- Coccidioidomycosis, disseminated or extrapulmonary
- Cryptococcosis, extrapulmonary
- Cryptosporidiosis, chronic intestinal (>1 month's duration)
- Cytomegalovirus disease (other than liver, spleen, or lymph nodes), onset at age >1 month
- Cytomegalovirus retinitis (with loss of vision)^b
- Encephalopathy, HIV related
- Herpes simplex: chronic ulcers (>1 month's duration) or bronchitis, pneumonitis, or esophagitis (onset at age >1 month)
- Histoplasmosis, disseminated or extrapulmonary
- Isosporiasis, chronic intestinal (>1 month's duration)
- Kaposi sarcoma^b
- Lymphoid interstitial pneumonia or pulmonary lymphoid hyperplasia complex^{a,b}
- Lymphoma, Burkitt (or equivalent term)
- Lymphoma, immunoblastic (or equivalent term)
- Lymphoma, primary, of brain
- *Mycobacterium avium* complex or *Mycobacterium kansasii*, disseminated or extrapulmonary^b
- *Mycobacterium tuberculosis* of any site, pulmonary,^{b,c} disseminated,^b or extrapulmonary^b
- *Mycobacterium*, other species or unidentified species, disseminated or extrapulmonary^b
- *Pneumocystis jirovecii* pneumonia^b
- Pneumonia, recurrent^{b,c}
- Progressive multifocal leukoencephalopathy
- *Salmonella* septicemia, recurrent
- Toxoplasmosis of brain, onset at age >1 month^b
- Wasting syndrome attributed to HIV

^aOnly among children <13 years of age.

^bCondition that might be diagnosed presumptively.

^cOnly among adults and adolescents >13 years of age.

AIDS, Acquired immunodeficiency syndrome; HIV, human immunodeficiency virus. Modified from Centers for Disease Control and Prevention. (2014). *Revised surveillance case definitions for HIV infection—United States, 2014*. Atlanta: Centers for Disease Control and Prevention. *MMWR Morbidity and Mortality Weekly Report*, 63, 1. Available at: <https://www.cdc.gov/mmwr/preview/mmwrhtml/rr6303a1.htm>.

Clinically latent infection

As the primary infection resolves, CD4⁺ T-cell counts increase while viral particle levels decrease and reach a steady-state level. During this stage, the virus is actively replicating in lymphoid tissue destroying large numbers of T cells. However, because T-cell production keeps pace with the number of T cells destroyed, immune function is intact, and the individual is asymptomatic. This asymptomatic period may last several years before the development of clinical immunodeficiency. The CD4⁺ T cell count gradually decreases while the virus continues to replicate and produces high titers of virus in affected tissues. This clinically latent period lasts approximately 10 years on average. Some patients are rapid progressors, developing AIDS within 1 to 2 years, as opposed to others considered long-term non-progressors, who may remain symptom-free for longer than 20 years. The rate of disease progression is dependent on host immune response, host genetic factors, characteristics of the infecting virus, and preventive therapies or lifestyle changes.

Progression to acquired immunodeficiency syndrome

The continued replication of HIV-1, coupled with the elimination of host immune cells, leads to deterioration of the immune system. At this point, the infected individual begins to develop one or more of the various AIDS-defining illnesses (see Box 38.1). Some of the most common AIDS-defining conditions (Fig. 38.23) include *Pneumocystis jirovecii* pneumonia, viral hepatitis, Kaposi sarcoma, oral candidiasis (thrush), and recurring bacterial pneumonias. In certain locations in which multidrug-resistant *Mycobacterium tuberculosis* is prevalent, a *syndemic* has been suggested, in which the convergence of two or more diseases act synergistically to magnify the burden of disease. HIV infection essentially alters the transmission

Table 38.4 Human immunodeficiency virus case definition, 2014^a

Stage	Laboratory evidence	Clinical evidence
0	Negative HIV test within 6 months of diagnosis	
1	Laboratory confirmation of HIV infection and CD4 ⁺ T lymphocyte count ≥500 cells/μL or CD4 ⁺ lymphocyte count >26% ^b	None required (no AIDS-defining condition)
2	Laboratory confirmation of HIV infection and CD4 ⁺ T lymphocyte count 200–499 cells/μL or CD4 ⁺ lymphocyte count 14–25% ^b	None required (no AIDS-defining condition)
3 (AIDS)	Laboratory confirmation of HIV infection and CD4 ⁺ T lymphocyte count <200 cells/μL or CD4 ⁺ lymphocyte count <14% ^b	Or documentation of an AIDS-defining condition with laboratory confirmation of HIV infection
Unknown	Laboratory confirmation of HIV infection and no information on CD4 ⁺ T lymphocyte count or percentage	And no information on presence of AIDS-defining condition

^aDefinitions are intended for public health surveillance only and not as a guide for clinical diagnosis.

^bStaging based primarily on CD4⁺ lymphocyte count; percentage considered if count is missing

AIDS, Acquired immunodeficiency syndrome; HIV, human immunodeficiency virus.

Modified from Centers for Disease Control and Prevention. (2014). *Revised surveillance case definitions for HIV infection—United States, 2014*. Atlanta: Centers for Disease Control and Prevention. *MMWR Morbidity and Mortality Weekly Report*, 63, 1. Available at: <https://www.cdc.gov/mmwr/preview/mmwrhtml/rr6303a1.htm>.

dynamics of tuberculosis, increasing the susceptibility and progression of disease.

Laboratory diagnosis

Multiple methods are available for diagnosing HIV infection with several available specimen types (blood, serum, plasma, urine, or even saliva). Generally accepted procedures rely on repeated positive screening results (EIA) followed by a more specific supplemental test for confirmation (IFA detection and NAAT). The initial IgG-sensitive EIAs (first and second generation) identified antibodies directed against HIV, which are not detectable until 4.5 to 6 weeks following initial infection. Newer screening assays detect the presence of anti-HIV IgG and IgM (third generation) or a combination of both anti-HIV antibodies as well as the p24 antigen (fourth generation). These latter-generation assays reduce the interval between infection and detection (window period) to within 2 weeks. This earlier detection can identify patients in the acute stage and potentially reduce the spread of disease. In August 2013, the FDA approved the first rapid test, the Determine HIV-1/2Ag/Ab Combo assay (Alere, Waltham, MA), that simultaneously detects HIV-1 p24 antigens as well as HIV-1 and HIV-2 antibodies. The test does not distinguish between HIV-1 and HIV-2 antibodies. In 2013,

the FDA also approved the first home testing kit, OraQuick in-home HIV test (OraSure Technologies, Bethlehem, PA). Saliva is the testing specimen, and antibodies to HIV can be detected in 20 to 40 minutes without sending the specimen to a laboratory for testing. Individuals are cautioned that positive results must be confirmed by laboratory testing.

Initial laboratory testing should employ an FDA-approved fourth-generation assay that detects HIV-1 and HIV-2 antibodies and HIV-1 p24 antigens (HIV1/2 antigen/antibody immunoassay). Reactive specimens should be tested using an FDA-approved supplemental antibody immunoassay to discriminate HIV-1 from HIV-2 antibodies. Specimens that test negative on the confirmatory assay should be subjected to an HIV-1 NAAT to assess them for possible acute HIV infection. This 2018 CDC recommended algorithm shortens the window period of HIV infection detection, accurately distinguishes between HIV-1 and HIV-2 infection, and reduces the turnaround time for confirmation of infection. The CDC does not have published guidelines for POC testing but does provide a recommended algorithm of a series of two different POC assays. If the first assay gives a positive result, a second assay using a different method or produced by a different manufacturer would confirm a probable infection. The patient should then be referred for HIV care. Recent studies demonstrate the efficacy of such an algorithm in rapidly



Fig. 38.23 Acquired immunodeficiency syndrome (AIDS)-defining illness. **A**, Pneumocystis pneumonia in a patient with human immunodeficiency virus (HIV) infection. This methenamine silver stain of lung tissue obtained from a biopsy of a patient with HIV infection showing cysts of *Pneumocystis jirovecii* ($\times 1000$). **B**, Patient with HIV infection with intraoral Kaposi sarcoma (KS) of the hard palate secondary to AIDS. KS lesions typically appear flat and purple-red in color but may be larger nodular growths. **C**, Patient with HIV infection with oral thrush caused by *Candida albicans*. (A, Courtesy Dr. Edwin P. Ewing, Jr., Centers for Disease Control and Prevention, Atlanta, GA; B, Courtesy Dr. Sol Silverman, Jr., Centers for Disease Control and Prevention, Public Health Image Library, Atlanta, GA; C, Courtesy Centers for Disease Control and Prevention, Public Health Image Library, Atlanta, GA.)

diagnosing HIV infection and providing immediate initiation of counseling, HIV treatment, and determination of an individual therapeutic regimen.

Treatment

Once an infection is confirmed, the patient should see a health care provider to receive counseling and determine when to start HAART, even if he or she does not feel sick. Current treatment practice is to begin aggressive antiviral therapy when infection is diagnosed, even in the absence of symptoms. This helps achieve maximum benefit from the antiretroviral agents. Patients receive counseling to help them make lifestyle changes, if necessary, such as quitting smoking, reducing alcohol intake, and eliminating the use of illegal drugs. Patients are also encouraged to practice safe sex to avoid spreading HIV and acquiring other infectious agents.

Patients should be screened for tuberculosis, hepatitis, and other STIs so that appropriate prophylaxis (antimicrobial therapy or vaccination) can be provided to protect against infection. HIV-specific laboratory tests are requested to determine the patient's baseline viral load and CD4⁺ T cell count, as is genotypic or phenotypic testing of the patient's infecting HIV to determine appropriate HAART and overall efficacy.

Advances in HIV treatment have dramatically improved the prognosis for infected individuals. Before the era of HAART, a positive HIV test result implied an eventual downward spiral in the patient's health, culminating in full-blown AIDS and death. The goal of therapy is to prevent the immune system from being overwhelmed by the viral load and make opportunistic infections unlikely. HIV infection is treated with a combination of two or more drugs from different classes (Table 38.5). Since the development of HAART, the mortality rate in developed countries, where patients have excellent access to health care, has dropped substantially, so the population of patients with HIV increases annually. Infected individuals are leading more active lifestyles, with sometimes many years passing before the development of opportunistic infections and AIDS. This delay in progression has led many people to have a more casual attitude toward HIV infection status. Whether they are infected or not, they often continue their risky lifestyle behaviors, thinking that a cure will be found before they develop AIDS. Some individuals even consider an HIV-positive status as a mark of distinction.

Although HAART has been very effective overall, resistance to HAART is increasing because of nonadherence, which could become a significant health issue. Monitoring of patients may soon be required. As of 2022, five patients have reportedly been cured of HIV infection. These patients received bone marrow transplants from donor stem cells with a CCR5 mutation (CCR5Δ32). CCR5 is a coreceptor for HIV adhesion. Clearly, there is a need for more effective prevention programs in an effort to reduce the overall spread of disease.

Table 38.5 Recommended medicines for treatment of human immunodeficiency virus infection

Drug class	Drugs
Nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs)	Abacavir, emtricitabine, lamivudine, tenofovir, zidovudine
Nonnucleoside reverse transcriptase inhibitors (NNRTIs)	Doravirine, etravirine, efavirenz, nevirapine, rilpivirine
Protease inhibitors (PIs)	Atazanavir, darunavir, fosamprenavir, ritonavir, saquinavir, tipranavir
Fusion inhibitors	Enfuvirtide
CCR5 antagonists	Maraviroc
Integrase strand transfer inhibitors	Cabotegravir, dolutegravir, raltegravir
Attachment inhibitor	Fostemasavir
Post-attachment inhibitor	Ibalizumab-uiyk

Modified from Food and Drug Administration (FDA) and the National Library of Medicine. Drug Information from the DailyMed website. *HIV and AIDS: Medicines to help you*. Available at: <https://www.fda.gov/consumers/free-publications-women/hiv-and-aids-medicines-help-you>.

Case check 38.5

HIV infection is not exclusively an STI, but it is strongly recommended that when an individual is suspected or confirmed to have an STI, she or he should simultaneously be screened for HIV infection. This is because many of the risk factors for HIV infection are similar to those associated with other STIs. The Case in Point demonstrates the correct procedure to follow for HIV screening for the young man. It is imperative that individuals who engage in sexual activities, especially those who have sexual relations with multiple partners, be screened often and also when they change partners. Many individuals may be unknowingly infected and spread STDs to their contacts.

Other sexually transmitted diseases

Case study

A 16-year-old adolescent female patient presents to the pediatric clinic for a routine sports physical. During the checkup, her physician discusses common teenage sexual infections and diseases noted in her community. Although she is not sexually active, her physician discusses the effects of HPV infection, noting that teenagers are more susceptible to HPV infection (40%) and also more likely to develop precancerous growths, which, in turn, may develop into invasive cancer. Her physician provides information about a vaccine for protection against nine of the most common types of HPV that cause cervical cancer and genital warts and recommends that she receive the vaccine.

Genital warts

Epidemiology

Genital wart, caused by HPV, is the most common viral STI in the United States and the most common STI in individuals 24 years of age or younger. According to the CDC, in 2018, approximately 42.5 million people in the United States were infected with HPV. An estimated 13 million individuals become infected annually. The disease is so common that it is estimated that nearly all of the sexually active population contracts HPV at some point in their lives. Over 40 different genotypes of HPV infect the genital tract. They are distinguished into two groups. Certain genotypes cause various forms of cancer in the genital area, in particular in the cervix, while the second group causes genital warts. Two specific genotypes in this second group, types 6 and 11, are associated with approximately 90% of all genital wart cases. Anyone who is sexually active is at risk of infection.

Clinical manifestations

Humans are the only known reservoir for HPV. The virus affects skin and mucous membranes and causes development of warts in the moist and dry areas of the genitals, which can vary in size and shape. They are typically small bumps that can be raised or flat, single or in groups, small or large, and sometimes cauliflower shaped (Fig. 38.24). The manifestation of the cauliflower-shaped genital warts in moist regions is called **condylomata acuminata**. Genital locations include the vulva, vagina, anus, cervix, penis, scrotum, and groin or thigh. Infection is spread through intimate contact, usually during vaginal and anal sex. In MSM, anal

warts occur more often than genital warts. Depending on the genotype involved, infections could lead to cancer.

Diagnosis

Diagnosis of genital warts is clinically made on physical examination. Flat warts, which are not as visible, may be detected by applying a weak acetic acid solution to suspected areas of genital tissue; the solution causes infected areas to whiten, making them more visible. However, this is not a very sensitive means of diagnosis and is not recommended. Tissue biopsies may also be performed to confirm HPV infection.

Treatment

There is no permanent cure for HPV, and recurrences may be found on skin near the initial site. Several methods of treatment are available, including cryotherapy with liquid nitrogen, surgery (scissor excision, shave excision, curettage, laser, or electrosurgery), trichloroacetic acid, bichloroacetic acid, and various topical medications. In some cases, no therapy is used to see whether the warts will disappear on their own. Interferon- α can also be injected directly into the warts that have returned after removal using other traditional methods. OTC cures for warts should not be used near the genitals because severe irritation can occur. Sexual contact should be avoided until the warts have been treated and eliminated.

Lifestyle changes can reduce the risk of infection. Abstinence is the safest way to prevent an infection. Individuals should use safe sex measures, with barriers to prevent transmission, and limit the number of sexual partners. Additionally, women who have regular Pap tests can potentially detect infection earlier and get proper treatment. Three HPV vaccines

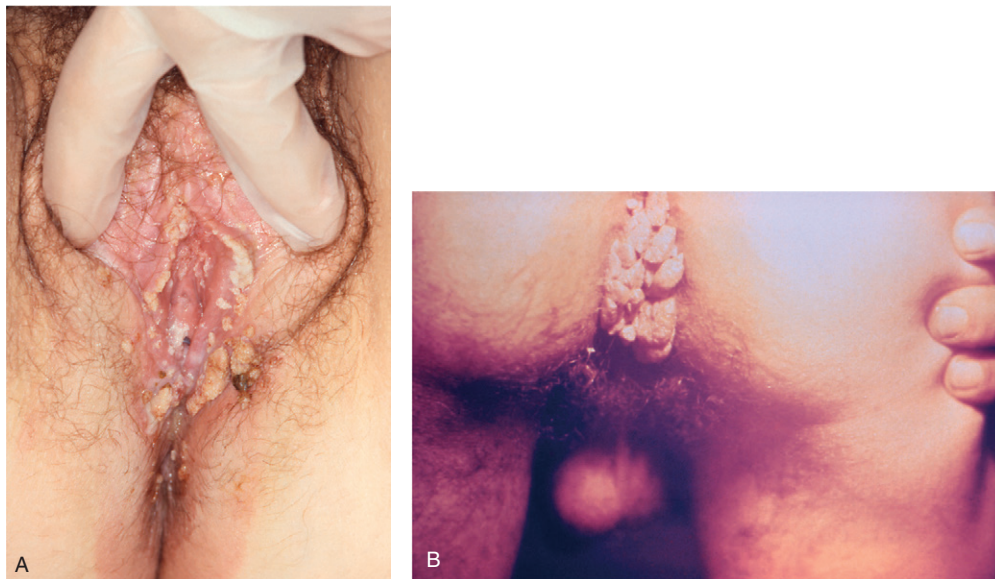


Fig. 38.24 **A**, Genital warts on the labia caused by human papillomavirus (HPV). **B**, Male patient with venereal warts (condylomata acuminata) in the anal region of the perineum caused by HPV. (A, Courtesy Joe Miller, Centers for Disease Control and Prevention, Public Health Image Library, Atlanta, GA; B, Courtesy Dr. Wiesner, Centers for Disease Control and Prevention, Public Health Image Library, Atlanta, GA.)

are licensed in the United States: Gardasil 9 and Gardasil (Merck, Kenilworth, NJ), and Cervarix (GlaxoSmithKline, Philadelphia, PA). Since 2016, Gardasil 9 is the only one currently available. It targets the common genotypes for genital warts (HPV 6 and 11), the most common genotypes that cause cervical cancer (HPV 16 and 18) and five low-risk types for cancer (HPV 31, 33, 45, 52, and 58).

The vaccine is approved for children and adults 9 to 26 years of age. Although not 100% effective, studies have demonstrated that HPV vaccines reduce the risk of disease and may substantially decrease public STI clinic workloads. However, a national survey conducted in 2018 found that only 70% of girls and 66% of boys 13 to 17 years of age received one or more doses of the HPV vaccine.

Case check 38.6

Infections by certain HPV serotypes are associated with genital warts and cervical cancer. The Case in Point illustrates the need for the girlfriend to receive the vaccination to protect her against both forms of the disease. One commercially available vaccine is also approved for use in males, so the young man might require counseling to determine whether he should also receive the vaccination series. The girlfriend had a sore throat, and a quick inspection revealed only redness. This case illustrates the need for more thorough questioning and patient history. If the girlfriend recently engaged in oral sex with her boyfriend, submission of a throat swab for culture or molecular testing might be appropriate.

Viral hepatitis

Epidemiology

Viral **hepatitis** is a major global health problem. On exposure to a hepatitis virus, the liver becomes inflamed, interfering with liver functions and possibly leading to chronic effects, such as cirrhosis, hepatocellular cancer or liver failure. There are eight recognized hepatitis viruses that affect humans, of which three—hepatitis A virus (HAV), hepatitis B virus (HBV), and hepatitis C virus (HCV)—can be sexually transmitted. HAV can be spread via any sexual activity with an infected person and is not limited to fecal-oral contact but is more common in MSM. HBV is spread via contact with blood, semen, vaginal secretions, and other body fluids of an infected individual (Fig. 38.25). Sexual transmission is the most common method of HBV transmission in the United States. HCV is spread by contact with an infected person's blood; sexual transmission of HCV occurs at a much lower frequency and is more often reported among MSM.

Worldwide, the WHO estimates that 1.5 million new cases of HAV infection occur annually and that 2 billion individuals have been infected with HBV overall, of which almost 300 million are chronic carriers of HCV. In the United States, although thousands of cases of viral hepatitis are reported to the CDC each year, an even larger number of cases are silent or go undiagnosed. According to the CDC, there are approximately 24,900 new HAV infections, 22,600 new HBV infections, and 50,300 new HCV infections each year. Of the infectious

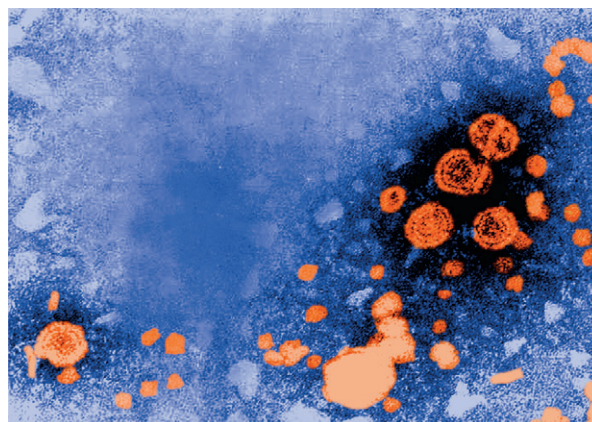


Fig. 38.25 Digitally colored transmission electron micrograph of hepatitis B virions. The large round virions are known as *Dane particles* ($\times 1,000,000$). (Courtesy Dr. Erskine Palmer, Centers for Disease Control and Prevention, Public Health Image Library, Atlanta, GA.)

diseases, hepatitis is considered a “silent killer” because it is often misdiagnosed and largely remains unrecognized by those infected. Worldwide, it claims three times more lives compared with HIV infection.

Clinical manifestations

The clinical manifestations of viral hepatitis are all similar, but many acutely infected patients are often asymptomatic, or their symptoms are so mild and self-limiting that they are hard to recognize. When symptoms are present, patients experience a short, mild, flulike illness, along with fatigue, nausea, vomiting, and diarrhea. Over time, they may recognize loss of appetite, unintentional weight loss, and abdominal pain. The hallmark feature of hepatitis is jaundice, a condition in which the patient's skin and the whites of the eyes appear yellow (Fig. 38.26). Their urine has a darker yellow coloration and their stools appear pale or clay colored.

Diagnosis

Initially, screening tests are performed to diagnose infection. An acute hepatitis panel is ordered, which typically includes tests for IgM anti-HAV, IgM antibody against hepatitis B core (anti-HBc), hepatitis B surface antigen (HBsAg), and anti-HCV antibody and HCV RNA. Presence of IgM anti-HAV is diagnostic for HAV infection. HBV testing relies primarily on two tests: detection of anti-HBc and detection of HBsAg. Antibodies developed against the core means that a patient has been previously infected with HBV. Detection of surface antigen indicates that HBV virions are circulating throughout the patient's bloodstream. In patients infected with HCV, HCV RNA is detectable before the appearance of anti-HCV. Additional serologic tests are used to identify the status of infection for HBV or HCV, such as acute or chronic infection, or whether or not an individual was previously vaccinated against HBV. Molecular testing determines the presence of viral nucleic acid and can be used as a confirmatory test for HCV infection. Viral load testing for HBV or HCV is intended to monitor the efficacy of antiviral therapy.



Fig. 38.26 Patient infected by hepatitis A virus with icterus, or jaundice of the conjunctivae and facial skin. (Courtesy Dr. Thomas F. Sellers, Centers for Disease Control and Prevention, Public Health Image Library, Atlanta, GA.)

Treatment

There is no specific treatment for HAV infection. Patients develop an acute infection, which is typically self-limiting and requires only supportive therapy. A vaccine against HAV is available and is recommended for those at greatest risk of disease. Acute infections with HBV are treated with supportive therapy. Chronic HBV infection and HCV infection are treated with antiviral agents. A vaccine against HBV is available, but there is none to protect against HCV infection. Chronic infections with HBV are treated with different antivirals, usually Interferon- α 2a (Roche, Switzerland) lamivudine, adefovir, telbivudine, tenofovir, or enfecavir. Liver transplantation may be an option for patients with severely damaged livers. Approximately 5% of all adults with HBV infection develop a chronic form of hepatitis, whereas younger children who are infected have a much greater rate of chronicity. For HCV infection, the Infectious Diseases Society of America recommends treatment with glecaprevir (8 weeks) or sofosbuvir (12 weeks). Approximately 90% of patients infected with HCV can be cured with therapy. In either case, patients with chronic infection require medical evaluation and regular monitoring of viral load to determine whether disease is progressing and to identify liver damage or hepatocellular carcinoma.

Pelvic inflammatory disease

Epidemiology

PID is a group of diseases affecting the female reproductive organs. It is a common complication of cervicitis in women who have mainly acquired the bacterial STIs chlamydia and gonorrhea. Annually, more than 1 million women are estimated to develop PID, and of these, over 100,000 may become infertile. PID develops as bacteria ascend the female genital tract into the reproductive organs. Sexually active women of childbearing age and women younger than 25 years of age are generally at the greatest risk of developing PID. Several risk factors for developing PID include having

multiple partners, frequent douching, and use of an intra-uterine device as a means of contraception. Previous episodes of disease also increase the risk of PID and further complications.

Clinical manifestations

The definition of PID is vague. It can be a conglomeration of several diseases of the female upper genital tract, such as endometriosis, salpingitis, tubo-ovarian abscess, and pelvic peritonitis. Disease can range from asymptomatic to severe. The full complement of commonly recognized symptoms of lower abdominal pain, fever, vaginal discharge, painful intercourse, pain on urination, and irregular menstrual bleeding should alert the health care provider to check for evidence of gonorrhea or chlamydia. Because symptoms may be vague and nonspecific, it has been estimated that PID goes unrecognized about two thirds of the time, leading to multiple complications caused by permanent damage to the reproductive organs.

Diagnosis

PID is very difficult to diagnose because of the absence of specific symptoms. There are no specific tests for PID, so the health care provider must usually make the diagnosis on the basis of physical examination and the clinical findings described earlier. Laboratory tests should be ordered to confirm infection with causative agents. Endometrial biopsy, transvaginal sonography, magnetic resonance imaging, or laparoscopy can also be used to ascertain the presence of findings consistent with PID. PID causes scarring in the reproductive organs, leading to serious complications, including chronic pelvic pain, infertility, ectopic pregnancy, and, in rare cases, spreading of the disease to the peritoneum and formation of scar tissue on the liver (Fitz-Hugh–Curtis syndrome).

PID can be prevented by adopting risk reduction methods. Abstinence or changes in social behavior and use of barrier methods all help decrease the probability of infection. When women identify any abnormal changes or recognize a potential infection, they should see their primary care provider immediately. The CDC recommends annual screening for all sexually active women 25 years of age and younger, all women in high-risk groups (e.g., multiple partners), and all pregnant women. The military recommends screening women for chlamydia at the entry level and until 25 years of age to reduce the level of PID in its female service members. Individuals with positive confirmed results can then be treated immediately, and their partners can be treated as well to reduce the risk of disease spread. Sexually active women experiencing pelvic or abdominal pain without an identifiable cause or demonstrating cervical motion, uterine tenderness, or adnexal tenderness should receive treatment.

Treatment

PID is treated with antimicrobial agents that are active against the specific causative organisms. Broad-spectrum



Fig. 38.27 Lesions of molluscum contagiosum on the leg and buttocks of a patient. These lesions appear as raised, pearlike papules or nodules that become umbilicated as they mature. (From Habif, T. [2009]. *Clinical dermatology: a color guide to diagnosis and therapy* [5th ed.]. London: Mosby.)

antimicrobials are used in an effort to promote action against multiple infectious agents. The regimen should include agents active against *N. gonorrhoeae* and *C. trachomatis*. Patients should be monitored after treatment to ensure treatment effectiveness. In severe cases, patients may need to be hospitalized for therapy.

Molluscum contagiosum

Molluscum contagiosum is a common skin disease caused by the molluscum contagiosum virus. This poxvirus can easily be spread through direct skin-to-skin contact or through contact with contaminated objects. It is considered an STI when found in the genital area. It is a common manifestation in patients with HIV infection and may signal immunosuppression. A common symptom is a small umbilicated papule that can become red and inflamed (Fig. 38.27). These papules can be easily removed when scratched or rubbed, thus spreading infection to other areas of the body. Diagnosis is clinical and can be confirmed by microscopic analysis. Treatment involves removal of the papules by scraping, freezing, or laser therapy. Avoidance of sexual contact is recommended until the infection has completely resolved.

In July 2022, monkeypox, a virus related to molluscum contagiosum virus, was declared a Public Health Emergency of International Concern by the WHO. In their August 7, 2022 report, 27,814 laboratory confirmed cases and 11 deaths were reported in 89 countries or territories. The United States reported about 11,000 cases at the same time. MSM seem to be at greatest risk for infection. This virus is spread by intimate contact as well as by contact with respiratory secretions and inanimate objects. Patients can present with a rash that may be located on or near the genitals (penis, testicles, labia, and vagina) or anus and could be on other areas like the hands, feet, chest, face, or mouth.

Epididymitis

Epididymitis is inflammation of the epididymis affecting men, usually those 19 to 35 years of age. This is generally a complication of chlamydia or gonorrhea. Common signs and symptoms include scrotal inflammation, testicular pain and tenderness, pain on urination or intercourse, chills and fever, lymphadenopathy, penile discharge, or blood in semen. Men who engage in high-risk sexual behaviors and have previously had STIs are at great risk of developing this condition. Diagnosis is based on clinical findings and Gram-stained smears of urethral secretions, positive leukocyte esterase urine test, or >10 WBCs per high-power field. Antimicrobial agents are administered for the confirmed infectious agent.

Proctitis

Proctitis is an inflammation of the rectal lining and is most common in those who engage in anal or oral-anal intercourse. Proctitis is mainly caused by the STIs chlamydia, gonorrhea, syphilis, and HSV infections. Symptoms include rectal bleeding, mucus passage, anal and rectal pain, and pain with bowel movements. When patients present to the health care provider for these complaints, additional STI screening should be performed. Treatment with antibacterial or antiviral agents can then be provided on the basis of the confirmed infectious agent.

POINTS TO REMEMBER

- STIs are caused by a wide variety and diverse group of infectious agents, including bacteria, viruses, protozoa, epizoa, and fungi.
- Many patients can have concurrent STI-related organisms. Therefore it is recommended to perform an HIV test on all patients suspected of having an STI.
- Syphilis testing requires positive nontreponemal and treponemal test results before infection is confirmed. Some facilities may perform the tests in reverse order.
- Clinical manifestations and presentations of the diseases are varied; some may be described as exudative, while others produce non-exudative lesions.
- Some STIs are asymptomatic.
- STIs are the main preventable cause of infertility in women.
- The epidemiology and pathogenesis of each infection may serve as useful tools in providing guidance with regard to specimen collection, transport, and handling, as well as methods for identification.
- The method and site of collection, quality of the clinical specimen, and clinical presentation are all important factors to consider when determining the causative agent of STIs.
- Molecular testing methods demonstrate increasing sensitivity of detection. By using noninvasive sampling, such as voided urine, more patients and contacts will present for screening assays, with the potential to reduce the sequelae of long-term infection through earlier treatment.
- HAART, when properly prescribed and used, can improve the quality of life and increase the longevity of those infected with HIV.

LEARNING ASSESSMENT QUESTIONS

1. What are the signs and symptoms and long-term effects of pelvic inflammatory disease (PID)?
2. What are the different causes of vulvovaginitis and how can they be distinguished clinically?
3. Why is it important to trace contacts of sexual partners of an individual with a diagnosis of a sexually transmitted infection (STI)?
4. Why is it important to screen for additional STIs in addition to that for which the patient is presenting?
5. What are the screening and confirmatory tests that aid in the diagnosis of STIs?
6. Which methods of prevention can be undertaken to reduce the overall spread of STIs?
7. What are the differences in clinical presentations and causes of chancroid and syphilitic chancre?
8. How has the advent of molecular testing advanced the detection of various STIs?
9. What are the distinguishing characteristics between gonococcal urethritis and nongonococcal urethritis (NGU)?
10. What genotypes are used in the Gardasil 9-valent human papillomavirus (9vHPV) vaccine and for what purpose?
11. Once HIV infection has been confirmed in an individual, which tests must be performed as these patients are managed?
12. Why are infections with hepatitis B virus (HBC) and hepatitis C virus (HCV) also listed as STIs?
13. Which important features allow herpesviruses (HPVs) to become latent infections?
14. For several years, the reported syphilis infection rates were declining, but now syphilis is beginning to make a comeback in the gay community. What could be causing this?
15. Some strains of *N. gonorrhoeae* have shown resistance toward penicillins, cephalosporins, fluoroquinolones, and some macrolides. What effects will this have on potential treatment regimens in the future? How may this affect screening tests for patients with probable gonorrhea infections?

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Infections in special populations

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CHAPTER OUTLINE

Healthcare-associated infections, 976
Ventilator-associated pneumonia, 976
Malignancy, 977
 Infections in patients with neutropenia, 977
 Infections in patients with cancer, 977
 Infections in patients with Hodgkin disease, 978
Acquired immunodeficiency syndrome, 979
Complement deficiency, 979
Burns, 979
Organ transplantation, 979
Postsplenectomy sepsis, 980
Cystic fibrosis, 980
Diabetes, 981
Aging, 981
Pregnancy, the fetus, and the neonate, 981
Bibliography, 982

OBJECTIVES

After reading and studying this chapter, you should be able to:

1. Describe the various conditions that compromise the host's immune status.
2. Discuss the various infections that occur in each population.
3. Determine the risk factors associated with each condition.
4. Associate the various infectious agents that affect special populations with the conditions that predispose these patients to a specific infection.

KEY TERMS

Acquired immunodeficiency syndrome (AIDS)
Adaptive immune response
Cell-mediated immunity (CMI)

Complement
Congenital infections
Endogenous
Exogenous

Granulocytopenia
Healthcare-associated infection (HAI)
Hodgkin disease (HD)
Human immunodeficiency virus (HIV)
Humoral-mediated immunity (HMI)
Immunocompetent
Immunocompromised
Immunosenescence
Immunosuppression
Innate immune response
Neutropenia
Opportunistic
Perinatal
Postsplenectomy
Septicemia

Case in point

A 32-year-old female patient was vacationing out of town when she was involved in a serious car accident. She had extensive bruising on her abdominal region. She was sent by Medevac helicopter to a trauma hospital, where an emergency splenectomy was performed. Surgery and postoperative recovery were uneventful, and the patient was discharged and returned home. Two weeks later, the patient had flulike symptoms, such as fever, malaise, epigastric discomfort, shortness of breath, and nonproductive cough. She was rushed to the hospital closest to her home. The attending health care provider noted a large scar above the patient's abdomen and immediately ordered laboratory tests. She was admitted to the hospital, and laboratory tests suggested that the patient had sepsis. Cultures of her blood and sputum grew *Streptococcus pneumoniae*.

Issues to consider

After reading the patient's case history, consider:

- Characteristics of the organism causing the infections
- Risk factors for acquisition of the organism
- Risk factor that made the patient immunocompromised
- Opportunistic organisms associated with immunosuppressed patients

The presence of certain types of underlying disease can make people more susceptible to infection. People can also be more susceptible to infection as a result of being very young

or very old, pregnancy, malnutrition, or trauma. All of these situations have in common some form of **immunosuppression** that makes the host more susceptible to infections. The organisms found in these situations are often **opportunistic** organisms. Opportunistic organisms are those that typically do not cause disease in an **immunocompetent** host. Bacteria, fungi, parasites, and viruses can all cause opportunistic infections. Bacteria, such as the normal skin microbiota (e.g., *Corynebacterium*), which are often regarded as skin culture contaminants from immunocompetent patients, might need to be fully identified, and an antimicrobial susceptibility test might need to be performed when bacteria are isolated from immunocompromised patients. Table 39.1 lists the major organisms isolated from immunocompromised hosts.

Many of the organisms listed in Table 39.1 are common in the environment or are members of the host's normal microbiota. Thus opportunistic infections can have an **endogenous** or **exogenous** source. An endogenous infection occurs when a member of the host microbiota invades a body site where it is not normally found and causes disease. For example, viridans streptococci, normally found in the oral cavity, can gain access to the bloodstream and cause endocarditis when the patient has poor dental hygiene or when a dental procedure that disrupts the normal mucous membrane barrier is performed. In an exogenous infection, an organism present in the environment or another source, such as another person, enters the body through inhalation, ingestion, or traumatic inoculation and causes disease. For example, fungal conidia present in the soil are inhaled and may cause a respiratory tract infection.

The normal host immune response, whether it is non-specific barriers to infection such as intact skin and mucous membranes, the **innate immune response** of which macrophages and neutrophils are a major part, or the **adaptive immune response** in which B lymphocytes and T lymphocytes are active, is usually able to eliminate most of the organisms. When one or more parts of the immune response are deficient, however, organisms that are normally cleared start to proliferate and cause disease.

The term **immunocompromised** is used to describe patients who have a disease or condition that affects the immune system, making them highly predisposed to infections by various opportunistic bacterial, fungal, parasitic, and viral pathogens. The number of immunocompromised patients has increased steadily during the past 30 years, reflecting increased use of immunosuppressive therapy, instrumentation, and organ transplantation. Unfortunately, the potential gain in years of useful life for many patients through successful management and treatment of diseases can be offset by serious adverse effects on the immune system. As a result, infection, rather than the primary illness, becomes the leading cause of death in immunocompromised patients.

The primary predisposing factor in these patient populations is the presence of underlying disease that affects host defense mechanisms. Examples of compromised defense mechanisms include the following:

- Leukocyte number or function, as in aplastic anemia and leukemia
- Humoral- and cell-mediated immune functions, as in B-cell and T-cell abnormalities
- Reticuloendothelial system function, as in splenectomized patients

Table 39.1 Opportunistic pathogens most often identified in the compromised host

Bacteria	<i>Staphylococcus aureus</i> <i>Staphylococcus</i> spp., coagulase negative <i>Streptococcus pneumoniae</i> <i>Streptococcus</i> spp., viridans group <i>Pseudomonas aeruginosa</i> <i>Escherichia coli</i> <i>Klebsiella pneumoniae</i> <i>Salmonella</i> spp. <i>Serratia</i> spp. <i>Burkholderia</i> spp. <i>Acinetobacter</i> sp. <i>Stenotrophomonas</i> sp. <i>Haemophilus influenzae</i> <i>Legionella pneumophila</i> <i>Nocardia</i> spp. <i>Listeria monocytogenes</i> <i>Clostridioides difficile</i> <i>Mycobacterium tuberculosis</i> <i>Mycobacterium avium</i> complex and other nontuberculous mycobacteria
Parasites	<i>Toxoplasma gondii</i> <i>Strongyloides stercoralis</i> <i>Cryptosporidium</i> Microsporidia
Fungi	<i>Candida</i> spp. <i>Aspergillus</i> spp. <i>Pneumocystis jirovecii</i> <i>Cryptococcus neoformans</i> <i>Histoplasma capsulatum</i> <i>Fusarium</i> Mucormycoses (e.g., <i>Absidia</i> , <i>Mucor</i> , <i>Rhizopus</i>) Phaeohyphomycoses (e.g., <i>Alternaria</i> , <i>Curvularia</i> , <i>Exophiala</i>)
Viruses	Herpes simplex virus Varicella-zoster virus Cytomegalovirus Epstein-Barr virus Hepatitis B virus Adenovirus Papovaviruses Enteroviruses Influenza virus

Other underlying factors include burns, diabetes mellitus, renal failure, and autoimmune diseases. Table 39.2 summarizes the various conditions that compromise the host's immune status and the pathogens usually encountered in infections associated with them. Infection with **human immunodeficiency virus (HIV)** and immunosuppressive therapy, such as chemotherapy and radiation

Table 39.2 Organisms associated with compromised patients

Immune status	Examples of conditions	Commonly encountered pathogen(s)
Reduced leukocyte number or function	Myelocytic leukemia Chronic granulomatous disease Granulocytopenia Acidosis Burns	<i>Staphylococcus</i> spp. <i>Serratia</i> spp. <i>Pseudomonas</i> spp. <i>Candida</i> spp. <i>Aspergillus</i> spp. <i>Nocardia</i> spp. <i>Legionella</i> spp. <i>Mycobacterium</i> spp.
Reduced humoral-mediated immune response and complement deficits	Lymphocytic leukemia Multiple myeloma Nephrosis Antimetabolite therapy Hypogammaglobulinemia	<i>Streptococcus pneumoniae</i> <i>Haemophilus influenzae</i> <i>Streptococcus pyogenes</i> <i>Pseudomonas</i> spp. <i>Pneumocystis jirovecii</i> Enteroviruses
Reduced cell-mediated immune response	Hodgkin disease Steroid therapy Uremia Antimetabolite therapy Acquired immunodeficiency syndrome Malnutrition	<i>Mycobacterium</i> spp. <i>Candida</i> spp. <i>Coccidioides immitis</i> <i>Histoplasma capsulatum</i> <i>Blastomyces dermatitidis</i> <i>Pneumocystis jirovecii</i> <i>Cryptococcus neoformans</i> <i>Cryptosporidium</i> Herpesviruses Adenoviruses Cytomegalovirus <i>Toxoplasma gondii</i> <i>Legionella</i> spp.
Reduced reticuloendothelial system function	Splenectomy Chronic hemolysis	<i>Streptococcus pneumoniae</i> <i>Salmonella</i> spp.

Modified from Drew, W. L. (2004). Infections in the immunocompromised patient. In K. J. Ryan & C. G. Ray (Eds.), *Sherris medical microbiology: an introduction to infectious diseases* (4th ed.). New York: McGraw-Hill.

therapy, particularly in organ transplant recipients, frequently lead to an immunocompromised condition. Secondary factors that facilitate infection include the use of invasive devices, such as indwelling intravenous (IV) catheters that breach the usual skin and mucosal protective barriers and introduce organisms into the bloodstream. One or a combination of any of these underlying conditions leaves a person less able to cope with infections.

Healthcare-associated infections

Hospitalized patients are at risk for several different types of infections for a variety of reasons, including being immunocompromised and undergoing invasive procedures. Collectively, these infections are referred to as **healthcare-associated infections (HAIs)** or *nosocomial infections*. A few of these infections are discussed separately in this chapter, such as ventilator-associated pneumonia (VAP). Central line-associated bloodstream infections, catheter-associated urinary tract infections, surgical site infections, and *Clostridioides difficile*-associated diarrheal disease are other examples. The risk of developing an HAI differs depending on the patient's immune status, overall health, medical conditions, and the procedures he or she is undergoing.

According to 2019 data from the Centers for Disease Control and Prevention (CDC), approximately 1 in 31 hospitalized patients develops at least one HAI. In 2002, it was estimated that 1.7 million HAIs occurred, with almost 100,000 deaths.

By 2015, there were 687,000 documented HAIs, with 72,000 deaths—a rate that, although still very high, was a significant improvement. Most HAI deaths are attributed to pneumonia and bloodstream infections. The most common organisms causing HAIs include *C. difficile* (70% of gastrointestinal [GI] infections), *S. aureus* (methicillin-susceptible *S. aureus* [MSSA] and methicillin-resistant *S. aureus* [MRSA]), enterococci (including vancomycin-resistant enterococci [VRE]), members of the Enterobacterales (particularly *Escherichia coli* and *Klebsiella* spp.), and *Legionella* spp. in cases of hospital-acquired pneumonia. Percentages of these pathogens depend on the geographic location and the type of facility (i.e., hospital or nursing home). Many of these isolates exhibit multidrug resistance. MRSA is the most common of these, but there has been a steady, worrisome increase in the past 10 years in the isolation of multidrug-resistant (MDR) gram-negative bacilli, including extended-spectrum β -lactamase (ESBL)-producing and carbapenem-resistant Enterobacterales. The good news is that overall, HAIs have dramatically decreased and continue to decrease slowly but steadily, with the implementation of specific infection control measures.

Ventilator-associated pneumonia

VAP is a nosocomial pneumonia that occurs in patients who have been on a mechanical respirator for more than 48 hours. It is the most common life-threatening nosocomial infection

in intensive care units. In 2019, the CDC HAI Progress Report listed a total of 24,724 ventilator associated events. Being on a mechanical ventilator substantially increases the risk of developing hospital-acquired pneumonia. VAP is estimated to occur in 5% to 67% of mechanically ventilated patients, depending on criteria used. In the United States, the incidence of VAP ranges between 2 and 16 episodes per 1000 ventilator days. As a result of aggressive preventive strategies, the mortality rate from VAP, which historically ranged from 33% to 50%, decreased in 2017 to 5.8% in early-onset VAP and 10.6% in late-onset VAP.

Signs of VAP include fever, white blood cell counts greater than $12 \times 10^9/\text{mL}$, and appearance of new or progressive pulmonary infiltrates or purulent tracheobronchial secretions. Although no gold standard exists, the diagnosis of VAP is usually made by using a combination of clinical, physiologic, radiologic, and microbiological evidence. The American Thoracic Society and the Infectious Diseases Society of America 2016 guidelines recommend obtaining noninvasive lower respiratory tract samples (e.g., endotracheal aspirates) for semi-quantitative microbiology culture. Collection of at least two sets of blood cultures may be helpful in determining the cause of the VAP in a febrile patient if the results of the cultures are positive. Rapid tests from additional types of specimens, such as urine for *Legionella* and *S. pneumoniae* antigens, serum for galactomannan or β -D-glucan for *Aspergillus*, and nasal specimens for respiratory syncytial virus (RSV) antigen, can be performed as well.

Colonization of endotracheal tubes with microbiota, usually environmental or oropharyngeal organisms that migrate downward into the pleural cavity, is the main cause of VAP. Organisms associated with VAP include *S. pneumoniae* and other streptococci; *Haemophilus influenzae*; *S. aureus*; *Legionella*; *P. aeruginosa*; *Klebsiella*; *Enterobacter*; *Serratia*; *Acinetobacter*; *Stenotrophomonas*; *Burkholderia cepacia*; fungi; and RSV. Frequently these infections are polymicrobial. Although oropharyngeal organisms are primarily seen in early-onset VAP, MDR pathogens are seen more frequently in late-onset VAP, and the associated mortality rate can be as high as 60%.

VAP has been reported as a major complication in patients who are hospitalized with COVID-19. Because of the deterioration of lung function caused from infection by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), many patients, especially older adults, immunocompromised individuals, and others with underlying health issues (e.g., diabetes, high blood pressure, obesity, heart and lung conditions), developed acute respiratory distress syndrome and required mechanical ventilation. COVID-19 patients were far more likely to develop VAP than patients without COVID-19 (28/1000 ventilator days vs. 13/1000 for patients without COVID-19). Preliminary reports indicate that the pathogens seen in these patients are generally the same as those associated with any other VAP, though one study reported a few cases of invasive aspergillosis and herpes virus activation in COVID-19 patients only. It is not yet known how often lung function is permanently damaged by serious infection; it is possible that some COVID-19 survivors could become a new group of immunocompromised patients.

Malignancy

A variety of deficiencies in **humoral-mediated immunity (HMI)**, involving B cells and antibodies, and **cell-mediated**

immunity (CMI), involving T cells, occur in patients with malignancies. Deficiencies can be caused directly by the tumor cells because of the cell type that has become malignant (e.g., leukemias and lymphomas, tumors of the immune system that are more associated with defects in the host immune response compared with tumors of other cell types). Immunosuppressive molecules (cytokines) secreted by the tumor cells can also produce immunosuppression. In addition, cytotoxic drugs administered to patients with cancer to kill the tumor cells contribute substantially to the breakdown of mucosal barriers and decrease CMI in these patients, further suppressing their immune response.

Granulocytopenia, or a reduction in granulocytes to 500 cells/mL or below, is commonly demonstrated in patients with hematologic malignancies and in those receiving chemotherapy. Once the granulocyte count drops below 500/mL to 1000/mL, the risk of infection increases steadily with the degree and duration of immunosuppression. In patients with a neutrophil count below 500/mL, the mortality rate may be as high as 60%. Almost every patient whose neutrophil count is less than 100/mL for 3 weeks or longer will develop an infection. The mortality rate among patients whose neutrophil count is lower than 100/mL during the first week of infection may be as high as 80%.

In addition to the decrease in the number of neutrophils, inadequate neutrophil function, including the inability to migrate to sites of inflammation; impaired phagocytosis; and reduced killing of ingested organisms predisposes patients with chronic leukemia and **Hodgkin disease (HD)** to infections. Most of these infections are caused by bacteria of endogenous origin; that is, GI tract, mucosal, or cutaneous biota. Fungi are also likely to invade the host with granulocytopenia.

Infections in patients with neutropenia

Septicemia often occurs in patients with **neutropenia** (decrease in the number of circulating neutrophils) because neutrophils play an important role in containing infections to a local site. *E. coli*, *K. pneumoniae*, and *P. aeruginosa* account for most infections with gram-negative bacilli. *S. aureus*, coagulase-negative *Staphylococcus*, and *Enterococcus* are the gram-positive organisms most commonly involved in sepsis. Less common pathogens that cause disease in the neutropenic patient include the gram-negative bacteria *Stenotrophomonas*, *Acinetobacter*, *Legionella*, *B. cepacia*, and *Campylobacter* and the gram-positive viridans streptococci. The risk of invasive fungal disease, often caused by *Candida* or *Aspergillus*, increases with time in neutropenia.

Pneumonia, especially when caused by gram-negative bacilli, is a major problem for the patient with neutropenia. Because the patient cannot mount an adequate immune response, pulmonary infection may spread rapidly and extensively. Infections with these organisms are particularly serious because they cause extensive necrosis and have a high fatality rate. Because of the lack of an inflammatory response to infection, patients with neutropenia do not develop the characteristic symptoms of pneumonia, and their infections may remain undiagnosed until after they have become disseminated.

Infections in patients with cancer

In general, cancer patients experience nearly three times the rate of infections as patients without cancer. Factors

responsible for the high incidence of infections among patients with cancer differ depending on the underlying malignancy. For example, tumors that outgrow their blood supply can become necrotic and infected. A GI tumor may ulcerate, providing a focus for invasion by enteric pathogens. Tumors may also obstruct the drainage of the tracheobronchial tree or urinary tract, permitting infection distal to the obstruction to become established. In addition, some cancers can inhibit the immune response, and chemotherapy and radiation treatment can cause neutropenia.

The mortality rate for patients with cancer who have sepsis is approximately 18% to 45%. In various types of cancers, such as lung cancer, the tumor burden is often the leading cause of death. However, infections account for approximately 20% of the deaths. In patients with lung cancer, pneumonia and sepsis are the most common fatal infections. The use of a totally implantable venous access port to deliver medication to patients with cancer has also been shown to slightly increase the risk of infection, such as sepsis. The two most common agents were *Candida* spp. and coagulase-negative staphylococci.

Skin infections are common in patients with cancer for several reasons. These patients often develop decubitus ulcers when they are bedridden for a long time. They also develop catheter-associated infections, cellulitis, and local necrosis involving the extremities, sometimes caused by venipunctures, IV lines, and other procedures.

Patients with hematologic malignancies and those who have undergone surgery for tumors involving the head and spine may experience central nervous system (CNS) infections. Gram-positive organisms are frequently seen in CNS and other infections in patients with cancer. Cancer patients develop meningitis caused by different organisms more often than patients without cancer; for example, more staphylococcal infections occur likely because of surgical manipulations, and there are fewer infections with community-acquired organisms such as *S. pneumoniae*, *H. influenzae*, and *Neisseria meningitidis*.

Fungal agents, such as *Aspergillus* spp. and *Mucor* spp., are associated with brain abscesses that occur in patients with leukemia. Oropharyngeal candidiasis occurs in about 5% of patients with cancer. Superficial GI candidiasis is also found in patients with acute leukemia and lymphoma. Although candidiasis can involve any portion of the GI tract, it is usually found in the esophagus and the stomach. Other areas sometimes affected include the kidneys, liver, spleen, and lungs. *Pneumocystis jirovecii* accounts for as many as 45% of cases of interstitial pneumonia in patients with cancer.

Other types of infections that occur in patients with cancer include those caused by viral and parasitic agents. The most serious viral infections are caused by members of the herpesvirus group. Normally, these viruses infect young patients, resulting in life-long immunity. Such immunity contributes toward maintaining latent infections that occur in immunocompetent individuals. The lack of cellular immunity, especially in immunosuppressed patients with cancer, results in reactivation of the latent virus and the potential for serious disseminated disease.

Herpes zoster, or shingles, occurs on reactivation of latent varicella-zoster virus (VZV). The CDC reports that approximately 1 in 3 individuals will develop herpes zoster in their lifetime. One study, however, reported a 1.9% higher rate of

VZV infection in patients with solid tumors and a 4.8% higher rate in patients with hematologic malignancies compared with the general population. The increased risk of infection is caused primarily by suppression of CMI; disease also correlates with T-cell suppression. Solid tumors do not typically affect immune response; however, systemic chemotherapy does adversely affect the immune response and frequency of zoster infection. Disseminated VZV that spreads to the lungs, liver, pancreas, adrenal glands, and CNS occurs in only 2% of the general population, but up to 35% in cancer patients. The mortality rate from VZV (zoster) infection is about 5% to 15%, and death is usually associated with pneumonitis. Current studies on how varicella vaccination in childhood (available since the 1990s) and zoster vaccination in older adults (available since 2006) affects the incidence and mortality of disseminated VZV in cancer patients will likely change these numbers.

Herpes simplex infections of the lip (cold sores or fever blisters) can result in extensive cellulitis because of superinfection with mixed bacterial and fungal organisms (especially *Candida* spp. and gram-negative bacilli) in patients with cancer. Children with cancer are more susceptible to cytomegalovirus (CMV) infection compared with adults. The most common manifestation is pneumonia that can be unilateral or bilateral and is usually accompanied by a more severe bacterial or fungal infection. Dissemination of CMV affects the lungs, kidneys, lymph nodes, heart, adrenal glands, spleen, pancreas, and bone marrow. Death sometimes occurs as a result of myocarditis, renal failure, or adrenal insufficiency.

Toxoplasma gondii is an obligate intracellular parasite that affects patients with malignancies. Infections may represent reactivation or primary exposure. *T. gondii* causes pneumonia, chorioretinitis, and a mononucleosis-like infection. It is seen most commonly in HD but also occurs in patients with lymphoma and leukemia.

Strongyloides stercoralis, an intestinal roundworm, has been reported in patients with chronic lymphocytic leukemia and lymphoma. Serious manifestations caused by *Strongyloides* infection can occur in patients receiving adrenal corticosteroids or antitumor therapy. Ulceration may result when the rhabditiform larvae penetrate the GI tract. Larval migration to the lungs may cause a pulmonary infiltrate. Interestingly, in many cases, the *Strongyloides* infection is not symptomatic and is not diagnosed until the patient becomes immunosuppressed.

Infections in patients with Hodgkin disease

Historically, HD has been associated with impairment of CMI. Patients with HD are especially susceptible to infections caused by facultative intracellular organisms, such as *Mycobacterium tuberculosis*, *Listeria monocytogenes*, and *Cryptococcus neoformans*, as well as viruses such as VZV. Intracellular organisms are able to multiply within the phagocytic cells and are often resistant to bactericidal and fungicidal agents.

As the number of circulating lymphocytes decreases, patients with HD who are undergoing chemotherapy become more susceptible to infections. Chemotherapy inhibits inflammation, reduces capillary permeability, and decreases cellular exudation. It also interferes with diapedesis of leukocytes, inhibits antibody production, and impairs reticuloendothelial function.

Acquired immunodeficiency syndrome

Acquired immunodeficiency syndrome (AIDS) is caused by HIV, a virus that infects and destroys CD4⁺ T-helper cells. The T-helper cells are critical for stimulating B cells to produce antibodies, cytotoxic T cells to lyse target cells, and monocytes/macrophages to phagocytize and destroy ingested organisms. Because of the integral role of T-helper cells in almost every facet of the immune response, the lack of this cell causes a profound immunosuppression that seriously undermines the body's ability to defend itself from infection and disease.

HIV infection, if not treated, can cause an infected individual to be susceptible to many different infections. HIV-infected individuals are particularly susceptible to infection by opportunistic pathogens and certain cancers. Some of the more common infections include *P. jirovecii* pneumonia, candidiasis of the respiratory tract, extrapulmonary *Mycobacterium tuberculosis* infection, *Mycobacterium avium* complex infection, cryptococcal meningitis, cryptosporidiosis or microsporidiosis with persistent diarrhea, toxoplasmosis encephalitis, CMV infection, herpes simplex virus (HSV) infection, and VZV infection. Kaposi sarcoma, caused by human herpes virus 8 (Kaposi sarcoma-associated herpesvirus); oral hairy leukoplakia, caused by Epstein-Barr virus (EBV); lymphoma of the brain in patients younger than 60 years of age; progressive multifocal leukoencephalopathy; and liver disease and liver tumors (especially if co-infected with hepatitis B or C virus) are also seen in HIV/AIDS patients. HIV/AIDS is discussed more fully in [Chapter 29](#).

Complement deficiency

The **complement** system is a series of proteins that, when activated, increase the activation and function of many different cells involved in the immune response. Complement is particularly important in the opsonization or coating of microorganisms that enhances phagocytosis of the organism by phagocytic cells, such as a neutrophil or monocyte/macrophage. Complement is also directly lytic for microorganisms.

Complement deficiency can be inherited or acquired and typically involves a lack of one particular protein of the system. Development of an autoimmune disease, such as systemic lupus erythematosus, which leads to the increased formation of immune complexes, can result in consumption of complement proteins. Deficiencies of a complement system protein result in increased susceptibility to infections, such as septicemia, pneumonia, or meningitis caused by organisms including *S. pneumoniae*, *H. influenzae*, *N. meningitidis*, members of the Enterobacterales, and staphylococci.

Patients with C3 deficiency are particularly susceptible to sepsis with pyogenic (pus-forming) bacteria, such as *S. aureus* or *Streptococcus pyogenes*. Deficiency in the terminal components of the complement cascade, C56789, results in an inability to lyse gram-negative bacteria, and patients are especially susceptible to developing recurrent bacteremia caused by *N. meningitidis*. Deficiency of the alternative complement pathway leads to serious *S. pneumoniae* infections, including meningitis.

Burns

An American Burn Association fact sheet from 2016 estimates that 486,000 burn injuries necessitating medical treatment, 40,000 hospitalizations, and 3275 deaths as a result of fire and smoke inhalation occur annually in the United States. Most deaths occur at the scene of the fire or shortly thereafter. Infection is a major cause of morbidity and death in those who survive. The risk of infection in a burn patient is directly proportional to the extent of the burn and reflects the combined effect of the impairment of all aspects of the host's defense system. As a result of systemic immunologic impairment, infection in sites other than the burn wound itself remains the most common cause of death among burn patients. Pneumonia is the most common infection seen in burn patients; burn wound infections have decreased in frequency because of vigilant wound care. The balance between host defense capacity and invasiveness of the microorganism in burn patients provides the optimal example of the susceptibility to infection in an immunocompromised patient.

In healthy individuals, bacterial competition (potential pathogens inhibited by the nonpathogenic resident microbiota) plays a significant role in controlling cutaneous colonization of skin. In burn patients, anatomic barriers have been breached, and members of the normal skin microbiota are destroyed or removed by desquamation. The denatured protein of the burn eschar and avascularity of the tissue provide an excellent environment for microbial growth.

The immune defense mechanisms in burn patients, both HMI and CMI, are suppressed. Immunoglobulin concentrations, especially those of immunoglobulin G (IgG), are depressed. Fibronectin levels are also reduced. Fibronectin, a dimeric α -glycoprotein found in plasma and the extracellular matrix of most tissues, is necessary for normal reticuloendothelial cell function and opsonizes *S. aureus*. A decrease in fibronectin levels precedes sepsis. The risk of fungal infections is increased with wound maceration, acidosis, lack of competitive bacterial pressure, and antimicrobial therapy. MRSA; *Enterococcus* (including VRE); *P. aeruginosa*; organisms in the Enterobacterales (increasingly MDR); *Acinetobacter*; *Candida* and other yeasts; *Aspergillus*, *Fusarium*, and *Mucor* spp.; and HSV are major causes of infections in burn patients.

Organ transplantation

The transplantation of solid organs and hematopoietic stem cells from bone marrow, peripheral blood, or umbilical cord blood has increased greatly over the past 30 years. In 2019, there were an estimated 152,863 solid organ transplants performed worldwide. In the United States, 36,000 organ transplantations were performed in 2020, an annual record for the eighth consecutive year. From 1988 to 2018, over 750,000 organs have been transplanted in the United States. To maintain the viability of the graft, immunosuppressive agents are given to organ transplant recipients to prevent immune-mediated destruction or rejection of the graft. Unfortunately, the immunosuppression is not limited to inhibiting the immune cells that recognize the engrafted organ. All cells of the immune system are adversely affected by these drugs, rendering the patient unable to mount a sufficient response to infection.

Organ transplant recipients can develop specific types of infections at certain intervals after transplantation occurs. During the first month after transplantation, postoperative bacterial infections are most common. Significant pathogens include antimicrobial-resistant organisms, including MRSA, VRE, *C. difficile*, and MDR gram-negative rods, such as ESBL-producing and carbapenem-resistant Enterobacterales and *Pseudomonas aeruginosa*. Approximately 1 to 6 months after transplantation, however, infections are caused by slower-growing pathogens, including fungi and viruses. These include *M. tuberculosis*, *L. monocytogenes*, *Nocardia*, *Aspergillus* spp., *Cryptococcus* spp., *Candida* spp., *P. jirovecii*, EBV, CMV, VZV, parvovirus B19, and polyomaviruses BK and JC. Infections with opportunistic organisms, including *Legionella* spp. in the lung and nontuberculous mycobacteria from catheters, occur as well. Bloodstream infections (BSIs) are some of the most common and deadly posttransplant infections, with a mortality rate of approximately 50% when combined with septic shock. The most common gram-negative organism causing BSI in posttransplant patients is *E. coli*, accounting for 36% of BSIs following solid organ transplants. The most common gram-positive organisms causing posttransplant BSI are staphylococci.

Immunosuppressive therapy reactivates latent CMV infection, which is probably the most significant cause of death and morbidity in transplant recipients. Without preventive therapy, 36% to 100% of organ transplant recipients develop CMV infection, and 11% to 72% are symptomatic, many in the first 3 months after transplantation and others in later phases. The direct effects of CMV include tissue infection presenting as pneumonitis, GI disease, hepatitis, and retinitis. Indirect effects linked to CMV after solid organ transplantation include increased risk of secondary infection, presumably because of immunosuppression, increased graft rejection, and accelerated coronary atherosclerosis following heart transplantation.

Infection caused by EBV in transplant recipients has also increased. EBV is recognized as a causative agent of B-cell lymphoma, the pathogenesis of which is related to immunosuppressive therapy. Furthermore, in patients who acquire primary EBV infection after transplantation, EBV-associated lymphoproliferative disease is likely to occur. Primary EBV infection usually develops in children; hence pediatric transplant recipients are at greatest risk. CNS infections caused by *L. monocytogenes*, *C. neoformans*, *T. gondii*, and *Aspergillus* spp. also occur in these patients.

Infections that occur more than 6 months after transplantation are usually associated with community-acquired organisms, such as those causing influenza, secondary bacterial pneumonia, foodborne illnesses (acquired during travel to locations with poor sanitary conditions), and mycotic infections from specific geographic areas that may result in dissemination. Precautions about travel to underdeveloped areas and unfamiliar places are given to transplant recipients to help them avoid unnecessary exposure to community-acquired infections.

Postsplenectomy sepsis

The major risk following splenectomy is overwhelming bacterial infection, or **postsplenectomy sepsis**, resulting from

the body's decreased ability to clear bacteria from the bloodstream and decreased levels of IgM in plasma. Mortality rates can be as high as 60%. Loss of splenic function puts patients at risk of infection from encapsulated organisms. *S. pneumoniae* is the most common, but infections can also occur from *H. influenzae*; *N. meningitidis*; non-encapsulated bacteria including *E. coli*, *Bartonella*, *Capnocytophaga*, and *Pseudomonas*; and the protozoan *Babesia*. The greatest risk of infection is in children, in patients with malignant or immunodeficient disease, and during the first 3 years after splenectomy. Patients should be aware that they are at increased risk of infection and seek medical advice at the onset of fever.

Case check 39.1

The patient in the Case in Point had a splenectomy following a traffic accident that made her particularly susceptible to infections from encapsulated organisms, including *S. pneumoniae*. The health care provider who treated her noted the surgical scar as well as her symptoms and ordered appropriate laboratory tests, including blood and sputum cultures, which helped the health care provider accurately diagnose her infection.

Cystic fibrosis

Cystic fibrosis (CF) is an autosomal recessive disorder caused by a mutation in the CF transmembrane conductance regulator (*CFTR*) gene, which codes for a transmembrane protein that controls the movement of chloride anions across the plasma membrane of cells. Normally, cyclic adenosine monophosphate (cAMP) regulates the transport of chloride across this membrane. The lack of *CFTR* function results in decreased chloride secretion and increased reabsorption of sodium and water by epithelial cells. The lack of water in the mucus causes it to be very thick and sticky. The thickness of the secretions adversely affects the function of the respiratory and GI tracts, sweat glands, and other exocrine glands.

The thick secretions in the lung, in particular, make the patient highly susceptible to chronic infections. According to the Cystic Fibrosis Foundation Patient Registry Annual Data Report of 2014, the most frequently isolated organisms from patients with CF are *S. aureus* (including MRSA) and *P. aeruginosa*. *S. aureus* is more common in children; in adults, *P. aeruginosa* is the dominant isolate, although the percentage of *P. aeruginosa* infections has steadily decreased in the past 15 years. In patients with CF, *P. aeruginosa* is more likely to be resistant to antimicrobial agents and forms a biofilm in a polysaccharide matrix that makes it harder for antimicrobial agents to reach the organism. Research has shown that the production of the polysaccharide and resistance to antimicrobial agents are linked genetically.

Other organisms isolated from the lungs of patients with CF include *H. influenzae*, *B. cepacia*, other nonfermenting gram-negative rods, *Aspergillus fumigatus*, and nontuberculous mycobacteria. Bacteria associated with lower respiratory tract infections in patients with CF often exhibit atypical colony morphology and grow more slowly. For example, *P. aeruginosa* isolates from patients with CF usually produce mucoid colonies from the production of a calcium alginate slime layer. *S. aureus*, commonly recovered from CF patients, can exhibit atypical colonial morphology in culture as well.

Infection with *B. cepacia* is associated with higher mortality and may affect eligibility for lung transplantation because of adverse outcomes after transplantation when the patient is infected by *B. cepacia* before transplantation.

Diabetes

In diabetes, there is insufficient production of insulin by the pancreas (type 1) or insulin resistance of cells (type 2), causing glucose not to enter cells to be used for energy. Because cells cannot take up glucose, body fluids contain increased levels of glucose. High levels of glucose lead to ketoacidosis, in which ketones and organic acids accumulate in blood, lowering the pH (termed *acidemia*). The high level of glucose is immunosuppressive, decreasing neutrophil function and CMI. The immune system suppression can be reversed by decreasing the blood glucose levels and increasing the pH. Infections that are more common in patients with diabetes versus patients without diabetes include the following:

- Pneumonia
- Pyelonephritis
- Soft tissue infections, especially of the extremities, often caused by *S. pyogenes* and *S. aureus*
- Mucocutaneous *Candida* infections
- Gangrenous cholecystitis

Infections that are almost exclusively seen in patients with diabetes include the following:

- Invasive otitis externa caused primarily by *P. aeruginosa*
- Rhinocerebral mucormycosis caused by *Rhizopus*, *Mucor*, and *Absidia* spp.
- Emphysematous pyelonephritis (formation of gas in the kidney)

Aging

Diverse changes in immune function occur with normal aging. Age-induced alterations of the immune system are often more qualitative (e.g., involving lymphocytic function) than quantitative (e.g., involving cell number or immunoglobulin levels). **Immunosenescence**, in which naïve T cells are no longer available to be stimulated by antigen, leads to the decline of the immune response over time. Therefore adults older than 50 years of age are not as likely to mount robust antibody responses to vaccines as well as younger adults. In addition, older adults might be immunosuppressed because they have undiagnosed underlying disease, are taking immunosuppressive medications for a diagnosed disease, or are malnourished. Furthermore, sometimes maintaining personal hygiene standards becomes poorer with aging, and many older adults may be in group housing conditions, both of which increase exposure to infectious microorganisms.

Infections, autoimmune diseases, and malignancies increase among older adult patients because of immunologic decline. Organ function declines with age, and the presence of underlying disease, especially malignancies and diabetes,

which are found more common in older adults, increases the susceptibility of older adults to infection. Infections in older adults usually occur in the respiratory tract (e.g., pneumonia, aspiration pneumonia) or urinary tract (especially catheter-related and enlarged prostate-related infections), skin and soft tissues (infection of pressure ulcers), abdominal cavity, or endocardium. Bacteremia of unknown origin can also occur.

The organisms most often isolated as pathogens in older adults are *S. pneumoniae*, *H. influenzae*, *Moraxella catarrhalis*, and *S. aureus* in cases of pneumonia and enteric gram-negative bacilli, *E. coli* in particular, in other sources. Infections with *Streptococcus agalactiae* (group B *Streptococcus*), *Listeria*, and VZV have increased in incidence and severity. A common and often deadly pathogen in older adults in nursing homes is *C. difficile*. In addition, the incidence of tuberculosis increases with age. Not only are older adults more likely to have serious infections caused by all pathogens when compared with younger individuals, but older adults are more likely to die of those infections. Older adults have a 5 to 10 times higher mortality rate caused by pyelonephritis and a 10-fold higher mortality rate caused by tuberculosis compared with younger patients. The mortality rate of pneumonia is at least three times higher in older adults than in younger patients. This was found to be particularly true during the SARS-CoV-2 pandemic, in which patients older than 50 years of age with COVID-19 disease had more serious symptoms, required ventilation more often, and died in far higher numbers than patients younger than 50 years of age; 95% of deaths have been in people older than 45 years of age.

Pregnancy, the fetus, and the neonate

Infections in pregnant women are of concern, not only to the patient but also to her fetus. Infections can cause preterm birth, spontaneous abortion, birth defects, and developmental disabilities in the offspring and can lead to increased morbidity and death for the woman. Pregnant women are naturally temporarily immunosuppressed so the antigenically different fetus is not rejected, leaving the woman more susceptible to infection and to more serious disease. The immune system in the fetus and neonate is immature, rendering them immunosuppressed as well.

The most common infection in pregnant women is a urinary tract infection, most often caused by *E. coli* and other gram-negative bacilli. Vaginal yeast infections caused by *Candida* spp. are also more common in pregnancy. Pregnant women with bacterial vaginosis are more likely to have premature rupture of the membranes or premature delivery, and the infant may then have a low birth weight because of the premature delivery.

Congenital infections (infections acquired by the fetus in utero) can be caused by the infectious agent crossing the placenta from the bloodstream of the infected woman. These infections can lead to developmental problems and death. Common causes include the following:

- *Toxoplasma gondii*
- Rubella virus
- CMV

- HSV
- VZV
- HIV
- Parvovirus B19
- Hepatitis B virus
- *Treponema pallidum*
- *S. agalactiae*
- *L. monocytogenes*

Other organisms can be transmitted during labor and delivery and in the **perinatal** period, and they are also associated with adverse outcomes (e.g., meningitis, blindness, brain damage) for the infant. Some of the organisms involved cause sexually transmitted diseases and are acquired as the infant moves through the infected birth canal. These agents include the following:

- *Neisseria gonorrhoeae*
- *Chlamydia trachomatis*
- *S. agalactiae*
- *E. coli*
- Human papillomavirus
- CMV
- HSV

POINTS TO REMEMBER

- Immunocompromised patients are susceptible to bacterial, fungal, and viral infections that are usually prevented by a healthy immune system.
- Most infections occurring in immunocompromised patients are caused by opportunistic pathogens of endogenous or exogenous origin.
- Defects in the immune system can be inherited or acquired.
- Successful treatment of infections in the immunocompromised patient depends on recognition of the deficit, early diagnosis, and prompt intervention.

LEARNING ASSESSMENT QUESTIONS

1. What is the most likely sequence of events that led to the febrile condition of the patient in the Case in Point?
2. What is the likely connection between infections and malignancy?
3. What hematologic conditions may predispose patients to various infections?
4. Which organisms are of concern in women who are pregnant?
5. Why are infections and malignancies common among older adults?
6. Which organisms are more likely to cause infections in patients with cystic fibrosis and ventilator-associated pneumonia?
7. Why do patients undergoing chemotherapy and radiation treatment have an increased risk of infections?
8. Infections caused by *Pneumocystis jirovecii* most often affect which of the following?
 - a. Upper respiratory tract
 - b. Lower respiratory tract

- c. GI tract
- d. Urinary tract

9. What is the greatest risk of infections to patients after a splenectomy?
 - a. Gastrointestinal infection
 - b. Skin infection
 - c. Pneumonia
 - d. Septicemia
10. What is the general cause of the increased risk of herpes zoster in patients with tumors?
 - a. Systemic chemotherapy
 - b. Immunosuppression caused by the tumor
 - c. Neutropenia caused by the tumor
 - d. All of the above

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Zoonotic diseases

David H. Nielsen and Lindsey E. Nielsen

CHAPTER OUTLINE

Transmission by scratches and bites from domestic or wild animals, 986

Pasteurellosis, 986

Erysipeloid, 986

Capnocytophaga canimorsus infection, 987

Cat scratch disease, 987

Transmission by direct contact or inhalation, 988

Anthrax, 988

Tularemia, 989

Brucellosis, 990

Leptospirosis, 991

Transmission by arthropod vectors, 992

Plague, 992

Lyme borreliosis, 993

Rickettsia infection, 994

Anaplasmataceae infection, 996

Trypanosomiasis, 997

Viral zoonotic diseases, 997

Emerging zoonoses, 998

Bibliography, 999

OBJECTIVES

After reading and studying this chapter, you should be able to:

1. Identify the animal hosts and/or vectors associated with the following infections:
 - Anthrax
 - Plague
 - Erysipeloid
 - Leptospirosis
 - Tularemia

- Lyme borreliosis
- Ehrlichiosis and anaplasmosis
- Trypanosomiasis
- Rabies

2. Correlate the pathogen (genus and species) that causes the diseases presented in this chapter.
3. Discuss the epidemiology and the clinical manifestations associated with each disease condition.
 - Anthrax
 - Plague
 - Erysipeloid
 - Leptospirosis
 - Tularemia
 - Lyme borreliosis
 - Cat scratch disease
 - Human granulocytic anaplasmosis
 - Human monocytic ehrlichiosis
 - Trypanosomiasis
 - Rabies
4. Compare the modes of transmission to humans for the following human rickettsial diseases.
 - Rocky Mountain spotted fever
 - Rickettsialpox
 - Murine typhus
 - Louseborne typhus
 - Scrub typhus
5. Describe the characteristics required for a disease to be classified as an emerging zoonotic infection.
6. Justify the need for an accurate patient history to help diagnose zoonotic infections.
7. Analyze the cause of the recent increase in the number of outbreaks attributed to emerging zoonotic infections, the worldwide cost associated with these over the past decade, prevention strategies, and the roadblocks associated with these prevention strategies.

KEY TERMS

Anthrax	Leptospirosis
Brill-Zinsser disease	Morulae
Buboes	Pneumonic plague
Bubonic plague	Rocky Mountain spotted fever (RMSF)
Emerging zoonoses	Tache noire
Erythema migrans (EM)	Tularemia
Eschar	Undulant fever
Human granulocytic anaplasmosis (HGA)	Weil syndrome
Human monocytic ehrlichiosis (HME)	Woolsorter disease
	Zoonoses

Case in point

A 16-year-old female resident of western Colorado experienced pain in the left axilla and arm, with numbness in the arm. The patient developed chills and fever 1 or 2 days later and had multiple episodes of vomiting. She went to the emergency department of a local hospital. The examination found that she had a normal temperature, elevated pulse (100 beats per minute), normal blood pressure, and normal chest x-ray. She reported falling from a trampoline 4 days earlier and was diagnosed with a possible brachial plexus injury related to this incident. The patient was treated with analgesics and given an appointment with a neurologist. Two days later, she was found semi-conscious at home and was taken to the emergency department. On examination, she was found to have a temperature of 39.1° C (102.5° F), reference range 35.6° C (96° F) to 37.5° C (99.5° F); pulse of 170 beats per minute, reference range 60 to 100 beats per minute; blood pressure of 130/70 mm Hg, reference range 90 to 119/60 to 79 mm Hg; altered mental status, neck pain, and generalized soreness. Less than 1 hour after arrival at the hospital, she experienced respiratory arrest and was intubated. Numerous gram-positive diplococci were found in a blood smear, and chest x-rays showed bilateral pulmonary edema. The patient was treated with 2 g ceftriaxone intravenously and transferred to a referral hospital. There she was diagnosed with septicemia, disseminated intravascular coagulation (DIC), acute respiratory distress syndrome, and possible meningitis. Sputum, blood, and cerebrospinal fluid (CSF) were taken for microbiological studies. A Gram stain of CSF revealed white blood cells (WBCs) but no bacteria. She was treated for gram-positive sepsis.

The patient's condition deteriorated rapidly, and she died later that day. Additional cultures of CSF grew unidentified gram-negative rods and *Streptococcus pneumoniae*. In addition, cultures of blood and respiratory aspirates yielded a gram-negative rod initially identified as *Yersinia pseudotuberculosis* by a rapid microidentification method. The blood culture isolate was sent to the state reference laboratory for confirmation and was subsequently identified as *Yersinia pestis*. It was later revealed that an extensive prairie dog die-off had recently occurred adjacent to the patient's residence. Four of five family dogs and one of three family cats had high antibody titers to *Y. pestis* F1 antigen. Investigators concluded that the patient had been infected by direct contact with abscess material from her pet cat while caring for it.

Issues to consider

After reading the patient's case history, consider:

- Zoonotic infections are common; as many as 61% of organisms pathogenic to humans are zoonotic in origin.
- A key piece of information in a patient history is animal exposure.
- Although some zoonoses are asymptomatic or characterized by mild symptoms, others are associated with high mortality.
- A surge in some atypical infections could reflect a larger change has occurred in local animal populations.
- Detection of certain infections in humans, such as West Nile virus infection, anthrax, monkeypox, and avian influenza might indicate an emerging public health threat.

Zoonotic infections are those that can naturally jump from animals to humans. The term *zoonotic* was used first by a German physician in 1855. Infections can be transmitted from animals to humans through various routes, including vectors (mosquitoes, ticks, fleas), skin-to-skin contact, animal bites, inhalation of respiratory droplets, and ingestion of contaminated animal products. Many bacterial zoonotic infections are foodborne or waterborne. According to the Centers for Disease Control and Prevention (CDC), more than 6 of 10 infectious diseases in people are spread from animals. A new viewpoint and appreciation for the zoonoses appeared after a publication by Cleaveland et al. in 2001. In this article, the authors described 1415 microbial species that cause infections in humans. The data included 217 prions and viruses, 538 bacteria and rickettsiae, 307 fungi, 66 protozoa, and 287 helminths. Amazingly, 868 of these pathogenic agents (61%) are zoonotic. To further emphasize the importance of **zoonoses** to human health, of the 175 emerging infections defined, 75% are zoonotic. Clearly, most of the pathogenic agents described in this text are likely to be classified as zoonotic agents.

Some zoonotic infections occur normally in their host animal populations and are able to jump to humans following increased interaction because of animal migration or encroachment of humans into animal habitats. Occasionally, once these infections are introduced into a human population, the zoonotic pathogen can be transmitted from human to human, resulting in a sustained persistence of the agent in the human population. Some of these zoonotic agents include human immunodeficiency virus, influenza virus, Ebola virus, and severe acute respiratory syndrome (SARS)-associated coronavirus. Other zoonotic agents are transmitted from animals to humans, but the resulting disease is generally not contagious from one person to another, so humans essentially represent dead-end hosts. Some zoonotic infections of this type include West Nile virus infection, rabies, ehrlichiosis, bubonic plague, tularemia, brucellosis, leptospirosis, and Lyme borreliosis. Because there are numerous zoonotic agents and infections, this chapter summarizes three major zoonotic disease transmission routes—animal scratches and bites, direct contact or inhalation, and arthropod vectors—and gives bacterial examples for each.

Transmission by scratches and bites from domestic or wild animals

Pasteurellosis

Case study

Parents took their lethargic and irritable 6-month-old daughter to an emergency department. The patient had a low-grade fever and a nonerythematous nodule on the right upper arm, but no sign of rash or lymphadenopathy was present. Two shallow abrasions, possibly scratches from the family's pet cat, were seen near the nodule. The results of the patient examination and laboratory tests were as follows:

- Rectal temperature: 38.9° C (102° F) (reference range 36.1° C [97° F] to 37.5° C [99.5° F])
- Pulse: 200 beats per minute (reference range 100 to 160 beats per minute)
- Respiration: 60 breaths per minute (reference range 30 to 60 breaths per minute)
- WBCs: $2.2 \times 10^9/L$ (reference range 6.0 to $17.5 \times 10^9/L$)
- 57% polymorphonuclear neutrophils (reference range 15% to 45%); 20% band neutrophils (>10% is a critical action value)
- 12% lymphocytes (reference range 47% to 77%); 6% monocytes (reference range 1.4% to 22%)
- Hematocrit: 28% (reference range 30% to 39%)
- CSF: three red blood cells per microliter; 327 WBCs per microliter (reference range <5 per microliter)
- 75% polymorphonuclear neutrophils
- 12% lymphocytes
- Glucose: 48 mg/dL (reference range >60 mg/dL)
- Protein: 79 mg/dL (reference range <40 mg/dL)

Cause

Pasteurellosis is caused by *Pasteurella multocida*, a pleomorphic ovoid to filamentous gram-negative bacillus, about 0.5 to 1.0 μm in size. It can be a primary pathogen or secondary invader. It is pathogenic for a wide range of hosts and occurs in the oral cavities of most dogs and cats (see [Chapter 18](#)).

Clinical manifestations

Pasteurellosis occurs worldwide and encompasses a wide range of endemic diseases of fowl and nonhuman mammals. The most common manifestation of human pasteurellosis is wound and soft tissue infections primarily from the bites or scratches of dogs and cats. Cats are usually involved more often compared with dogs. Although animal bites are the most common source of *P. multocida* infection in humans, animals can also infect preexisting abrasions by licking them. The infected site becomes inflamed within 48 hours, but the presence of pus is rare. Typically, the patient with pasteurellosis is afebrile and does not have inflamed lymph nodes.

Other clinical manifestations include pericarditis, corneal ulcers, upper and lower respiratory tract infections (including epiglottitis, sinusitis, otitis media, and pneumonia), endophthalmitis, and genitourinary tract infections (including pyelonephritis and renal abscess). Rare complications of human infection with *P. multocida* have been reported. These include

sepsis, meningitis, septic arthritis, abscess, peritonitis, and osteomyelitis. The pathogenesis of *P. multocida* infection in humans is not fully understood, but the antiphagocytic capsule and an outer membrane antiphagocytic protein appear to play a key role in dissemination of the pathogen. Some *P. multocida* isolates, including some from humans, produce an exotoxin as well as sialidase, hyaluronidase, surface adhesions, and siderophores. Identification of *P. multocida* infection is based on, the clinical presentation, results of Gram-stained smears; bacterial cultures; biochemical tests; imaging studies, such as computed tomography, magnetic resonance imaging (MRI), and echocardiography; lumbar puncture; arthrocentesis; and paracentesis. Most *Pasteurella* spp. are susceptible to oral antimicrobial agents, including amoxicillin, amoxicillin-clavulanic acid, and fluoroquinolones.

Erysipeloid

Case study

A 67-year-old male patient experienced fever and lower back and bilateral leg pain after falling from a ladder. He went to the emergency department 10 days later and was admitted to the hospital. He was afebrile upon admission, but examination revealed a pruritic erythema of the trunk and extremities. The patient explained that the rash had started as a papule that had spread, showing central clearing. The patient also worked near hog pens. Blood cultures were collected, and routine laboratory tests were done. X-rays showed no bone damage, and the urinalysis was normal.

On hospital day 2, the laboratory reported gram-positive cocci isolated from the blood of the patient. The isolated organism was preliminarily identified as an α -hemolytic *Streptococcus*, but after reexamining the Gram-stained smear and performing biochemical testing, the organism was identified as *Erysipelothrix rhusiopathiae*. The patient was treated with penicillin.

From Gorby, G. L., & Peacock, J. E., Jr. (1988). *Erysipelothrix rhusiopathiae* endocarditis: microbiologic, epidemiologic, and clinical features of an occupational disease. *Reviews of Infectious Diseases*, 10, 317.

Cause

E. rhusiopathiae is the causative agent of the zoonosis erysipelas, seen primarily in pigs (rose disease), and is characterized by fever, skin lesions, arthritis, and sudden death. In humans, *E. rhusiopathiae* causes syndromes referred to as *erysipeloid of Rosenbach*, *erysipelothricosis*, *rose disease*, and *fish handler's disease*. *E. rhusiopathiae* is a thin, non-spore-forming, facultatively anaerobic, gram-positive bacillus, 0.2 to 0.4 $\text{m} \times 0.8$ to 2.5 μm , which can grow singly, in short chains, or in long filaments (see [Chapter 16](#)).

Epidemiology

Often isolated from contaminated water and soil, *E. rhusiopathiae* is important in veterinary medicine. It is found in swine, poultry, dogs, cats, horses, sheep, small mammals, fish, and crustaceans. The organism occurs worldwide. Swine erysipelas is an important economic disease in North America, South America, and Europe. Occupational exposure increases the risk of infection with *E. rhusiopathiae* in farmers, fishermen, butchers, and veterinarians.

Clinical manifestations

The most common clinical manifestation of infection with *E. rhusiopathiae*—erysipeloid—occurs as a result of handling infected animals or animal products. Three clinical manifestations may occur in humans. The first is a localized cutaneous form (also known as *erysipeloid of Rosenbach*) in which the site of infection is usually an abrasion or wound of the hands or fingers (Fig. 40.1). The infection is mild, localized, and self-limiting. An edematous lesion forms 2 to 7 days after infection. Regional lymphadenitis is present in about one third of the patients. Erythema and itching might also be present. The lesion usually heals without treatment within 1 month. The second type is a more diffuse cutaneous form, and the third is a generalized systemic infection with bacteremia. Patients with structural valvular disease, alcoholism, or other predisposing conditions may develop sepsis and endocarditis.

Capnocytophaga canimorsus infection

Case study

A 47-year-old female patient came to the emergency department with weakness, diarrhea, and a facial rash. The patient had a pulse of 80 beats per minute, reference range 60 to 100 beats per minute, and a temperature of 36.5°C (97.7°F), reference range 35.6°C (96°F) to 37.5°C (99.5°F). She had an eschar on the left hand, with no evidence of cellulitis. The facial rash covered her nose and cheeks. There were ecchymoses on her extremities. Her arms and legs were cold to the touch. Lung, cardiac, and central nervous system (CNS) function were normal. The patient was admitted to the intensive care unit.

The patient reported that a dog had bitten her on her left hand 5 days before admission. She had seen a local primary care provider shortly after the bite, and he treated her with corticosteroids for an allergic reaction. The patient received empiric amoxicillin-clavulanic acid and amikacin therapy, followed by ceftazidime and amikacin. The result of aerobic blood culture (one bottle, using Bactec NR-660) was positive, showing a thin, gram-negative bacillus. The blood was subcultured onto brain-heart infusion agar supplemented with 10% horse blood, and small colonies appeared after 48 hours of incubation in carbon dioxide. The laboratory identified the isolate as *Capnocytophaga canimorsus*.

From Hantson, P., et al. (1991). Fatal *Capnocytophaga canimorsus* septicemia in previously healthy women. *Annals of Emergency Medicine*, 20, 93.

Cause

C. canimorsus, previously known as *dysgonic fermenter 2* (CDC group DF-2), is a thin, nonsporing, nonmotile, oxidase- and catalase-positive, gram-negative bacillus, 1 to 3 µm long. The organism grows poorly on laboratory media. This pathogen causes a wide range of clinical manifestations, ranging from a mild, self-limiting localized infection to fulminant septicemia, with involvement of several organs. Infection commonly occurs as a result of handling dogs or cats. The carrier rate for this organism in dogs seems to be low, but inadequate recovery techniques may have influenced this finding.

Clinical manifestations

C. canimorsus is a normal inhabitant of canine and feline oral biota, and infections occur as the result of bites. Dissemination and septicemia occur more often than the self-limiting lesions. About 90% of infections are found in patients with underlying health issues, particularly those who are splenectomized, have

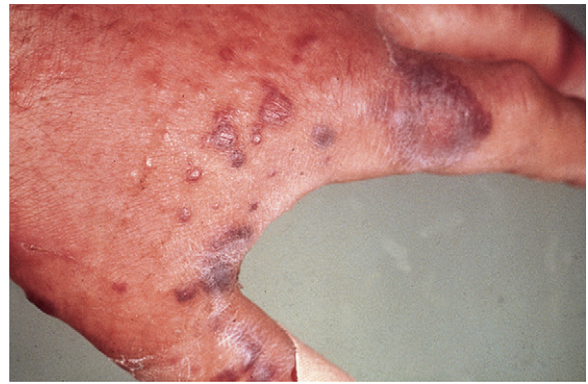


Fig. 40.1 Erysipeloid. An infection of the hands caused by *Erysipelothrix rhusiopathiae* is characterized by blue-red patches on skin. (From Conlon, C. P., & Snyderman, D. R. [2000]. *Mosby's color atlas and text of infectious disease*. London, United Kingdom: Mosby.)

cancer, or have a history of alcohol abuse or intravenous drug use. The most severe infections are seen in splenectomized patients, who develop an endotoxin-mediated Schwartzman-like phenomenon with purpura, septic shock, and DIC. Infection of previously healthy individuals is rare, probably because of the susceptibility of the organism to normal serum killing.

Cat scratch disease

Causes

Cat scratch disease (CSD), also known as *cat scratch fever*, is predominantly caused by the bacterium *Bartonella henselae*. There are currently about 60 species in the genus *Bartonella*. Approximately 50% of those species can cause disease in humans. Some species that infect humans (with vectors and/or animal hosts listed in parentheses) are *B. henselae* (flea, cat), *Bartonella clarridgeiae* (flea, cat), *Bartonella vinsonii* subsp. *vinsonii* (ear mites, voles), *Bartonella vinsonii* subsp. *berkhoffii* (ticks, dogs, coyotes), and *Bartonella vinsonii* subsp. *arupensis* (ticks, mice). *Bartonella bacilliformis* causes the diseases Oroya fever (acute form) and verruga peruana (chronic form). These diseases are not considered zoonoses because humans are the primary reservoir.

CSD was first described in 1889 and was first associated with cats in 1939, when the term *cat scratch disease* was first used. However, the causative agent of the disease was not determined until 1983. Researchers at the Armed Forces Institute of Pathology in Washington, DC, described coccobacilli on patient tissue sections processed using the Warthin-Starry silver stain. After almost a decade of study, the new pathogen was named *Afipia felis*. Serologic data and gene amplification tests were used to determine that most CSD infections were not caused by *A. felis* but rather by *B. henselae* (formerly known as *Rochalimaea henselae*), with some cases also caused by *Bartonella grahamii*. An estimated 12,500 cases of CSD occur annually in the United States, although the disease may be underdiagnosed. Infection is likely transmitted among cats and kittens via cat fleas (*Ctenocephalides felis*). However, human transmission is generally attributed to cat scratches, licks, or bites because almost all patients in whom CSD is diagnosed report exposure to felines, with most of them also recalling a scratch or bite by the feline.

Clinical manifestations

About 1 to 3 weeks after exposure to a cat, symptoms appear. About one half of the patients present with a small skin lesion

(papule) resembling an insect bite. It occurs at the inoculation site, typically on the hand or forearm. The lesion develops into a vesicle and may become a partially healed ulcer. The lesion can heal in a few days to a few weeks. In some cases, unilateral lymphadenitis develops about 3 weeks after exposure. Lymphadenopathy can persist for weeks or months, but often heals spontaneously. Patients may also experience flu-like symptoms, including fever, malaise, and anorexia. The disease in humans is usually mild and self-limiting, lasting anywhere from 6 to 12 weeks in untreated patients. Therefore conservative symptomatic treatment is recommended in patients with mild or moderate CSD.

Case check 40.1

As in the case of many diseases, access to complete patient history, including animal contact and occupational exposure, is the key to accurate diagnosis of CSD. Unfortunately, laboratorians often do not have access to medical records. One strategy for increasing access is by fostering a relationship with the health care provider, particularly the infectious disease staff. Such efforts are encouraged because they provide communication and increase the likelihood that laboratorians will have access to relevant patient information.

Transmission by direct contact or inhalation

Anthrax

Case study

A 57-year-old male patient, employed as an electrician, felt ill and feverish before reporting to the hospital and collapsed at home when he tried to stand. He had been bitten by an insect on the upper left chest the previous day while at work. He was taking no medications and had no travel history. Examination of the patient detected hypotension; a temperature of 38.2° C (101° F), reference range 35.6° C (96° F) to 37.5° C (99.5° F); and a necrotic lesion at the site of the insect bite on the chest, with edema and erythema.

Subsequent laboratory values were as follows:

- Hemoglobin: 17.9 g/dL (reference range 14 to 18 g/dL)
- WBCs: $10.9 \times 10^9/L$ (reference range 4.5 to $11 \times 10^9/L$)
- Platelets: $262 \times 10^9/L$ (reference range 150 to $400 \times 10^9/L$)
- Serum creatinine: 117 $\mu\text{mol/L}$ (reference range 65 to 119 $\mu\text{mol/L}$)
- Serum creatine phosphokinase: 883 IU/L (reference range 20 to 200 IU/L)
- Serum urea: 4.9 mmol/L (reference range 1.8 to 7.1 mmol/L)
- Antistreptolysin O: 240 Todd units (reference range <200 Todd units)

The patient was admitted to the hospital with a diagnosis of streptococcal cellulitis and septicemia and treated with intravenously administered penicillin G and flucloxacillin. The initial diagnosis was proven wrong when the blood culture samples taken at the time of admission became positive for *Bacillus anthracis*. Fortunately, the antimicrobials prescribed were appropriate, and the patient made a full recovery. Misdiagnosis occurred in this case largely because of the rarity of this disease and incomplete patient history. However, it was later determined that the patient had recently worked with untreated imported hides, which, coupled with the necrotic ulcer, would have increased the index of suspicion for anthrax.

From Mallon, E., & McKee, P. H. (1997). Extraordinary case report: cutaneous anthrax. *The American Journal of Dermatopathology*, 19, 79.

Cause

Anthrax, also known as **wool sorter disease** and *malignant pustule*, is caused by *B. anthracis*, a large ($2.5 \times 10 \mu\text{m}$), gram-positive, spore-forming bacillus. This organism occurs naturally in the soil and is a pathogen of herbivores, such as cattle, sheep, and goats. Human infections occur as the result of direct or indirect contact with animals or animal products.

Epidemiology

B. anthracis survives well in soil that is neutral or mildly alkaline. Areas with alternating dry and wet seasons enhance the development of anthrax. Floods tend to concentrate spores, which remain in the grasses after flood waters drain. These spores have been known to survive in fields for as long as 20 years. Animals are then infected by grazing in a contaminated area. Anthrax occurs worldwide, commonly in agricultural regions, such as Central and South America, Africa, and the Middle East. Anthrax is rare in the United States.

Recurrences of anthrax are prevented by containing the spores and eliminating their spread through the environment. Because the anthrax bacteria in tissues do not sporulate until they are exposed to oxygen, infected animal carcasses are usually incinerated whole or are buried in deep pits. Application of lye or quicklime, a practice that has been used to speed up the decay of carcasses, is no longer recommended because calcium ions are now believed to prolong anthrax spore survival. Vaccines are available for humans and for cattle. A cell-free vaccine, prepared from the protective antigen (PA) of *B. anthracis*, is used in individuals working in high-risk occupations. Another vaccine, made with an avirulent, nonencapsulated strain of *B. anthracis*, is available for animals.

Clinical manifestations

Human anthrax manifests in four forms: cutaneous, intestinal, pulmonary, or injectional. The route of transmission determines the incubation period and symptoms.

Cutaneous anthrax

The most common form of anthrax is the cutaneous form (99% of cases), which mimics many other cutaneous infections. It is most common in nonindustrialized countries. The spores enter the host through abraded skin and then germinate. After 48 to 72 hours, a papule is formed; it darkens and ruptures, leaving a painless, craterlike ulcer, which progresses to a necrotic **eschar**. The infection usually remains localized and self-limiting, and the eschar heals without scarring. In about 20% of patients with cutaneous anthrax, the immune system is unable to contain the infection, and *B. anthracis* enters the bloodstream and spreads. With treatment, death is rare.

Gastrointestinal anthrax

The gastrointestinal form, the second most common form of anthrax, is also found more frequently in nonindustrialized nations. It is caused by the ingestion of meat or meat products contaminated with spores. It presents in one of two forms: either as an oral or oropharyngeal form resulting in lesions on the buccal cavity, tongue, or pharyngeal wall; or the intestinal form, producing ulcers anywhere in the gastrointestinal tract. Once the spores are ingested, they germinate, and the organisms gain entry to the intestinal mucosa. Dissemination of the

bacteria can occur via the lymphatics. Clinically, the patient will be febrile and have bloody stools and can lose as much as 12 L of fluid per 24-hour period.

Pulmonary (inhalation) anthrax

The pulmonary form of anthrax is caused by inhaling spores, usually from contaminated animal products. This form of anthrax is more common in industrialized countries. Macrophages ingest the spores and concentrate them in lymph nodes. Eventually, the spores germinate, and sepsis occurs. Symptoms include fever, chills, malaise, and fatigue. This form of infection usually results in death of the patient, which, in the absence of treatment, can occur within 24 hours. The case fatality rate of inhalational anthrax is more than 85%. Administration of antimicrobial therapy and supportive care may reduce the fatality rate, but this treatment appears to be effective only when initiated before the onset of respiratory symptoms.

Injectional anthrax

Injectional anthrax occurs with the injection of drugs of abuse. Even though the route of infection is percutaneous, symptoms differ from those of cutaneous anthrax. Skin near the injection site may be bruised or discolored; however, papules and eschar characteristic of cutaneous anthrax are absent. In injectional anthrax, severe soft tissue infections with edema and necrosis are often present, and this can progress rapidly to septic shock and death. Patients are treated with antimicrobial agents and often with debridement or fasciotomy.

B. anthracis produces three virulence factors: (1) glutamic acid, which inhibits phagocytosis; (2) lethal factor (LF); and (3) edema factor (EF). For LF and EF to become biologically active toxins, they must first combine with the *B. anthracis* protective antigen (PA), which binds to the host cell. PA is also a transport protein, which carries EF and LF into the host cell. EF is an adenylate cyclase that causes characteristic edema. Necrosis occurs as the result of increased capillary permeability and destruction of the phagocytic cells.

Edema can be remarkable and may cause the patient to suffocate by literally swelling the neck shut. Excessive edema of the neck, thorax, and mediastinum signals the beginning of a rapidly fatal course. Several approaches may be considered in the diagnosis of anthrax, depending on the clinical presentation, including examination of the direct Gram-stained smear of tissue, nucleic acid amplification of tissue, chest radiography, computed tomography, blood and tissue cultures, and lumbar puncture. Treatment options include orally administered doxycycline, quinolones, penicillin, raxibacumab, obiltoximab,

and human anthrax immune globulin (Anthraxil) (Table 40.1). Doxycycline and ciprofloxacin may be used prophylactically in individuals who have been exposed to anthrax but have not developed symptoms.

Tularemia

Case study

A 63-year-old male patient noticed swelling and localized pain in the dorsum of his left thumb 5 days after a cat bite. He was treated with orally administered penicillin and cloxacillin for 3 days. He continued to experience pain, general malaise, fever, and vomiting and was admitted to the hospital. The patient was lethargic; his temperature was 38.7° C (102° F), reference range 35.6° C (96° F) to 37.5° C (99.5° F); his chest was clear; and he was hemodynamically stable, with a 3- × 5-cm indurated, erythematous region on the dorsal aspect of the base of his left thumb and hand. Because this was a possible abscess, the health care provider performed an incision and drainage, but no abscess was found. Swabs from the infected region grew coagulase-negative staphylococci.

The patient was treated with intravenously administered penicillin and cloxacillin. Five days after admission, the patient developed shortness of breath and had patchy pneumonic infiltrates of the middle and lower lobes of the right lung. Seven days after admission, lymphangitic streaking on the upper left extremity and tender axillary lymphadenopathy were detected. The patient's therapy was changed to clindamycin and gentamicin. The patient's fever abated, and his respiratory status improved.

A swab from the wound was plated onto sheep blood agar and chocolate agar plates and incubated in 5% carbon dioxide. After 3 days, small, smooth, gray colonies were visible. Gram-negative coccobacilli were identified as *Francisella tularensis* through fatty acid analysis and slide agglutination by using specific antisera. Acute-phase and convalescent-phase serum samples had titers of 1:800 and 1:3200, respectively. The patient's cats were adopted strays that had lived outdoors and had probably fed on wild rodents.

Cause

Tularemia is caused by *F. tularensis*, a strictly aerobic, gram-negative bacillus about 0.2 × 0.2 to 0.7 μm in size. The first isolation of the bacterium was in 1911 during an epizootic outbreak of plaguelike ground squirrel disease. The organism was named *Bacterium tularensis* after Tulare County, California, which was the site of the outbreak. In 1944, researchers defined the role of cottontail rabbits and jackrabbits in the transmission of the disease to humans. Since 1945, the proportion of vectorborne (*Dermacentor andersoni*, *Dermacentor variabilis*, and *Amblyomma americanum*) transmission has increased, whereas transmission from vertebrate reservoirs has decreased. In 1959, the genus name of the organism was changed to *Francisella* in honor of Edward Francis, who first isolated the organism.

Epidemiology

According to the CDC, in the United States, tularemia has been reported in all states except Hawaii. Approximately 240 cases of tularemia are reported annually. *F. tularensis* is found predominantly in the Northern Hemisphere. The two biovars *F. tularensis* biovar *tularensis* (type A) and *F. tularensis* biovar *palaeartica* (type B) occur in different parts of the world. In North America, the predominant biovar is type A, the strain that is more virulent in humans. Humans are usually infected

Table 40.1 Anthrax treatment and management

Clinical presentation	Treatment
Cutaneous anthrax	Oral doxycycline or quinolones
Nonbioterrorist anthrax, inhalational anthrax, anthrax meningitis	Penicillin
Bioterrorist anthrax	Doxycycline or quinolones
Inhalational anthrax or for prevention when alternative therapies are not available	Raxibacumab, Anthim (obiltoximab)
Inhalational anthrax in adults and children	Human anthrax immune globulin (Anthraxil) in combination with antimicrobial therapy

with this strain by rabbits or ticks, although more than 100 species of vertebrate and invertebrate natural reservoirs can transmit the infection. Type B occurs throughout the Northern Hemisphere but is more often recovered in Europe and Asia. It is less virulent than type A and is usually transmitted by rodents and mosquitoes. Types A and B differ from each other biochemically and genetically but not serologically.

Clinical manifestations

Tularemia is an acute, febrile, granulomatous disease characterized by rapid onset and flulike symptoms. The most common presentations are ulceroglandular (ulcers and lymphadenopathy), oropharyngeal (pharyngeal ulcer and lymphadenopathy), oculoglandular (conjunctival ulcer and lymphadenopathy), glandular (lymphadenopathy without ulcer), pleuropulmonary (no ulcer, possible lymphadenopathy), and typhoidal (no ulcers or lymphadenopathy). The ulceroglandular, oculoglandular, and glandular types usually occur as the result of direct contact with infected vertebrate or invertebrate reservoirs. Typhoidal and oropharyngeal cases of tularemia usually occur after eating contaminated food.

Pneumonic tularemia may result from exposure to aerosols. The symptoms associated with tularemia include fever, chills, headache, and myalgia. Typhoidal tularemia, especially when complicated by pneumonia, has a high fatality rate. In the United States, most cases of tularemia are of the ulceroglandular type. These infections are usually caused by direct contact with contaminated game or by insect bites. The incubation period of tularemia is typically 3 to 5 days. A papule, which forms at the site of infection, eventually ulcerates. Patients usually have only one lesion, although multiple lesions can occur. Because the disease is rare and

there are no characteristic symptoms, patients are often initially misdiagnosed. The actual site of the lesion is indicative of the transmission; lesions of the hands or arms often indicate infection by direct contact with infected mammals, whereas lesions on the head or back are suggestive of insect vector transmission. Streptomycin is the drug of choice to treat tularemia. Gentamicin and amikacin are acceptable alternatives.

Brucellosis

Causes and epidemiology

Brucellosis is referred to by many other terms, including *Mediterranean fever*, *Malta fever*, *Gibraltar fever*, *Bang disease*, *Neapolitan fever*, *Cyprus fever*, and **undulant fever**. The genus name comes from Sir David Bruce, who, in 1887, was first to describe these agents as the cause of undulant fever. In the United States, fewer than 100 cases of brucellosis are reported annually. Four species, which originate from animal reservoirs, are pathogenic to humans, in descending order:

- *Brucella melitensis* (goats)
- *Brucella suis* (swine)
- *Brucella abortus* (cattle)
- *Brucella canis* (canines)

Other species not known to cause human disease are *Brucella neotomae* (desert wood rat) and *Brucella ovis* (sheep). In animal hosts, brucellae can induce spontaneous abortion secondary to bacteremia. While the urine and milk of infected animals contain infective organisms, most animal infections are asymptomatic. *B. melitensis*, the most common agent of human brucellosis, occurs in many areas of the world, including Mexico, Central and South America, southeastern Europe, countries bordering the Mediterranean, Africa, southern Russia, India, Iran, and central Saudi Arabia.

Clinical manifestations

In the United States, brucellae infect humans primarily through eating undercooked meat or consuming unpasteurized (raw) dairy products. Contact with infected animals and animal products, including inhalation, can also lead to infection. Veterinarians, meat packers, shepherds, and abattoir workers have the highest risk of infection. The organism can enter the body through abraded skin, mucous membranes (e.g., gastrointestinal tract), or the conjunctivae. In experiments, *B. abortus* has even penetrated intact skin.

After an incubation period of 1 to 3 weeks, brucellae are disseminated hematogenously, where circulating monocytes ingest them. Brucellae are facultative intracellular parasites. Monocytes transport brucellae to lymph nodes. From there, the bacteria spread to the spleen and the liver. *B. abortus* usually causes granuloma formation, whereas *B. melitensis* usually causes formation of microabscesses. *B. melitensis* is also able to inhibit phagosome-lysosome fusion in phagocytic cells, allowing intracellular bacterial replication. Normal human serum is bactericidal to *B. abortus*, but not to *B. melitensis*, accounting for the relative differences in their pathogenicity. The symptoms of acute brucellosis are chills, fever, sweating, weakness, myalgia, and fatigue.

Case study

A 25-year-old male patient complaining of headache, malaise, arthralgia, and a 6-kg weight loss was admitted to the hospital. His symptoms started 3 months ago after his return from a trip to Syria. He reported having eaten fresh goat cheese. On admission, he was in moderate distress and had the following laboratory results:

- WBCs: $5.3 \times 10^9/L$ (reference range 4.5 to $11 \times 10^9/L$)
- C-reactive protein (CRP): 10.7 mg/dL (reference range $<1.0 \text{ mg/dL}$)
- Malaria smear: negative
- Serology for *Salmonella* spp.: negative
- Blood culture: positive for *Brucella abortus*
- Serology for *B. abortus*: positive, titer 1 : 10,000

The patient was treated daily with oral doxycycline, 400 mg, and rifampin, 600 mg. The patient's condition improved, he became afebrile, and his CRP level returned to normal after a few days. The patient's girlfriend developed the same symptoms 2 months later and was admitted to the hospital. Blood culture results were positive for *B. abortus*. She was successfully treated and recovered without complications.

The authors believed this to be the first case of possible sexual transmission of *B. abortus* in humans. The female had no travel history or other risk factors. The couple had unprotected sexual intercourse, and sexual transmission was considered to be the most likely route of infection.

From Thalhammer F., et al. (1998). Unusual route of transmission for *Brucella abortus*. *Clinical Infectious Diseases*, 26, 763.

Leptospirosis

Case study

A 48-year-old male patient, employed as a river dredger, presented with headache, fever, dark urine, arthralgia, diarrhea, breathlessness, and confusion, which had become worse over the past 4 days. The patient had hypoxia, and chest x-rays showed rapid deterioration, with shadowing in all regions of the lungs. He was treated with doxycycline and erythromycin. His condition continued to deteriorate, and he was given mechanical respiratory support. Methylprednisolone was prescribed in addition to the antimicrobials. Chest x-rays showed continued deterioration for a period of 7 days before showing gradual clearance. Renal function also returned to normal. He was discharged after 21 days. It was determined that the patient had culture-positive leptospirosis, with severe pulmonary hemorrhage. The date of the positive culture result was not reported.

Reviews of the Tropical Public Health Unit records showed 149 cases of leptospirosis. Of the 24 patients hospitalized, 5 had life-threatening pulmonary hemorrhage. Although not frequently reported, this complication is worth noting because of its serious consequences. In the authors' experience with the five cases, all patients gave nonspecific histories of malaise, myalgia, and fever. Renal impairment and jaundice, considered the hallmarks of leptospirosis, were absent in two of the five patients.

From Simpson F. G., et al. (1998). Leptospirosis associated with severe pulmonary haemorrhage in Far North Queensland. *The Medical Journal of Australia*, 169, 151.

Cause

Leptospire are spirochetes, about 6 to $20 \times 0.1 \mu\text{m}$ in size (Fig. 40.2). They are motile and have two subterminal flagella. The cells have characteristic hooks on the ends. The genus *Leptospira* contains a large group of serologically diverse organisms. Nearly 70 species are in the genus. As many as 250 serovars are organized into 23 serogroups.

Epidemiology

Until recently, **leptospirosis** was not considered a widely prevalent infection. Current thought suggests that this

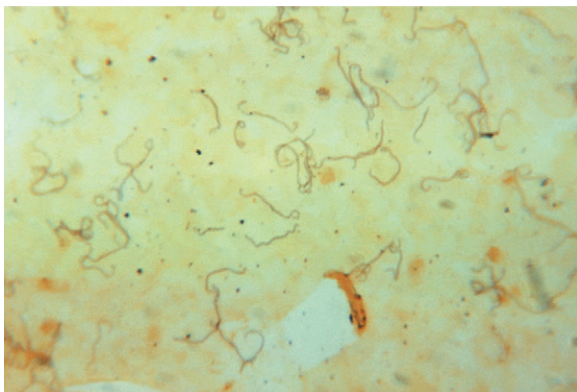


Fig. 40.2 Photomicrograph of *Leptospira* taken from a liver preparation in a patient with fatal leptospirosis (silver stain, $\times 1000$). (Courtesy Dr. Martin Hicklin, Centers for Disease Control and Prevention, Atlanta, GA.)

infection is one of the most common zoonoses, primarily as a result of exposure during recreational activities. It is most often reported in the United States from the southeastern, Gulf coast, and Pacific coast states and Hawaii and the U.S. territory Puerto Rico. About 100 to 150 cases of leptospirosis are identified annually, with the highest annual occurrence rate reported in Puerto Rico followed by Hawaii. Recreational activities that involve contact with water or moist soil are usually associated with transmission. In Hawaii, some homes have rainwater catchment systems, and these have also been reported as a risk factor. In as many as 5% to 10% of cases, leptospirosis is fatal.

Rodents and domestic animals are the primary reservoirs for the organism, although other animals, including cows, horses, mongooses, and frogs, can also harbor leptospires. Humans may be directly infected through contact with animal urine or indirectly by contact with soil or water that is contaminated by urine from infected animals. The usual portal of entry is via cuts or abrasions in the skin or along mucous membranes. The patient in the Case in Point worked as a river dredger and doubtless had frequent repeated contacts with untreated water. Infected humans can shed leptospires in urine for up to 11 months, infected cows can shed them for 3.5 months, infected dogs can shed them for 4 years, and infected rodents can shed them possibly during their entire lifetime.

Veterinarians, abattoir workers, fish and poultry processors, and dairy workers are at risk for leptospirosis. Agricultural workers and soldiers are also at risk because of contact with soil and mud. Leptospirosis is endemic in most areas of the world, although the incidence of disease might be underreported because of the difficulties associated with diagnosing infections. The infection is more prevalent in areas with warm climates, especially in late autumn and early winter.

Clinical manifestations

Leptospiral infections can range from subclinical to lethal. The organisms enter the host through mucous membranes or abraded skin. The incubation period ranges from 5 to 14 days. Most cases of leptospirosis are subclinical or mild. Anicteric leptospirosis is usually a biphasic disease. The first phase, known as the *leptospiremic* or *febrile phase*, typically lasts about 1 week. This phase is characterized by sudden temperature spikes, severe headaches, nausea, vomiting, and muscle aches. Patients often experience confusion secondary to dehydration. Additionally, most patients develop vivid pink eyes. During this period, leptospires can be recovered from the patient's blood and CSF.

The second phase of anicteric leptospirosis is referred to as the *convalescent* or *immune phase*. During this period, leptospires disappear from the circulatory system and CSF. This change occurs after the appearance of specific immunoglobulin M antibodies. Symptoms may subside for a few days, but a limited febrile episode may follow. Patients can also develop aseptic meningitis and severe headaches. During this stage of infection, the urine contains leptospires, but the blood of the patient does not. The length of this stage depends on the serotype of the infecting leptospire but is generally between 4 and 30 days.

Leptospira interrogans serovar *icterohaemorrhagiae* can cause icteric leptospirosis, also known as **Weil syndrome**.

This form of leptospirosis is more life-threatening than the anicteric form and is seen in 5% to 10% of all patients with leptospirosis. Weil syndrome starts in the same way as anicteric leptospirosis. Around the third day of the illness, however, the patient develops hemolysis, jaundice, and renal failure. These symptoms occur as the leptospires multiply in the liver and kidney. The mortality rate of Weil syndrome ranges from 5% to 15%. In fatal cases, renal failure is the usual cause of death. In nonfatal cases, as antibodies are produced, leptospires clear from the patient's kidneys, brain, and eyes. The treatment of choice for mild leptospirosis is doxycycline, ampicillin, or amoxicillin, and severe leptospirosis is treated with intravenously administered penicillin G.

Case check 40.2

Although many diseases that fall into the category of zoonotic may have pathognomonic symptoms, the patient's history, including animal contact and occupational exposure, is key to a timely and accurate diagnosis.

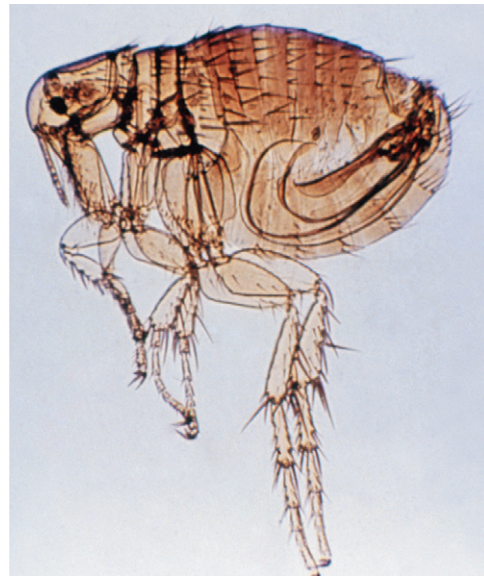


Fig. 40.3 Rodent fleas carry zoonotic infections, such as plague and murine typhus. (Courtesy Centers for Disease Control and Prevention, Atlanta, GA.)

Transmission by arthropod vectors

Plague

Cause

Plague is caused by the gram-negative bacillus *Y. pestis*. This genus is named for Alexandre Yersin, a French microbiologist who isolated the plague bacillus during an epidemic in Hong Kong in 1894. Historically, three pandemics have occurred; Black Death, the notorious second pandemic, caused 25 million deaths ($\approx 30\%$ of the European population). Rats are the natural host for the fleas that transmit the disease from one host to another by means of a bite, and humans are accidental hosts. Fleas (*Xenopsylla cheopis*) are the vectors (Fig. 40.3) that normally infest brown (*Rattus norvegicus*) and black (*Rattus rattus*) rats. In addition to rats, plague is also common in species included in the family Sciuridae such as prairie dogs (*Cynomys* spp.) and squirrels (*Sciurus* spp.).

The United States averages 7 human plague cases annually, with most cases occurring in the Southwest. The organism persists in this region through the sylvatic cycle, being passed among fleas and their natural hosts. *Y. pestis* can survive for months in animal burrows, and uninfected hosts can become infected from this reservoir. The risk of humans becoming infected with *Y. pestis* increases when human dwellings encroach on infested host habitat, when natural hosts are eradicated and fleas seek new hosts, or when living conditions are favorable to infested host cohabitation in rural or urban areas. Transmission to humans can also occur via domestic cats that hunt rodents.

Life cycle

Fleas develop yersiniosis when they take a blood meal from an infected animal host. Yersiniae multiply in the gut of the

flea, eventually reaching such a high concentration that they block the flea's gut. This blockage impairs the flea's ability to feed, and infection begins when the fleas regurgitate the plague bacilli into the bite wound while feeding. In response to a lack of nutrition, fleas infest a wider range of hosts including humans and will bite more often.

Clinical manifestations

Plague has two major manifestations: bubonic and pneumonic forms. **Bubonic plague** is an acute, febrile disease with an incubation period of 1 to 7 days. The disease is usually characterized by a lesion in a regional lymph node that drains the infected area. The resulting painful **buboes** usually occur in the groin, axillae, or subauricular area. In the Case in Point, the patient complained of a painful axilla and numbness in her arm. This may have been the beginning of bubo formation. Within 3 to 6 days after the onset of infection, the patient developed signs of septic shock. If untreated, the organism can be disseminated hematogenously, leading to septicemia. However, septicemic plague may occur without the bubonic form. The mortality rate of septicemic plague is almost 100%, with death occurring within 1 to 3 days.

Septicemic plague may lead to secondary pneumonia, referred to as **pneumonic plague**. Pneumonic plague is almost always the result of hematogenously spread bubonic or septicemic plague. There are two forms of pneumonic plague: primary and secondary. Primary pneumonic plague occurs when the patient acquires the organism via infectious airborne droplets; secondary pneumonic plague results from the plague bacillus entering the lungs of the patient who has either bubonic or septicemic plague. Pneumonic plague presents an increased risk of person-to-person transmission because respiratory droplets produced during coughing are extremely infectious. In the absence of respiratory precautions, this form may lead to localized or devastating outbreaks.

Lyme borreliosis

Case study

A 28-year-old female patient came to the emergency department with shaking chills and perspiration. She explained that before the fever she had swollen and painful ankles, knees, wrists, and elbows. Her temperature was 38° C (100.4° F), reference range 35.6° C (96° F) to 37.5° C (99.5° F), and synovitis was noted in the wrists, elbows, knees, and ankles. She had no rash or lymphadenopathy. She reported a family history of osteoarthritis and rheumatoid arthritis. The patient lived in a rural area and showed evidence of multiple insect bites, although she could not remember any recent tick bites. The patient was treated with naproxen and doxycycline for polyarthritis, systemic lupus erythematosus, and late-stage Lyme borreliosis.

Cause

Lyme borreliosis, caused by the spirochete *Borrelia burgdorferi*, is an arthropod-borne disease for which humans are accidental hosts (see [Chapter 23](#)). The disease is primarily transmitted by ticks, including *Ixodes affinis*, *I. dammini*, *I. scapularis* (the deer or black-legged tick), *I. pacificus* (western black-legged tick), and *I. ricinus* (the European sheep tick). *B. burgdorferi* organisms are gram-negative spirochetes about 0.18 to 0.25 $\mu\text{m} \times 4$ to 30 μm in size ([Fig. 40.4](#)).

Epidemiology

Lyme borreliosis, or Lyme disease, is the most common vector-borne disease in the United States, with approximately 30,000 cases reported annually. Lyme borreliosis exists throughout the world, with about 65,000 cases reported each year in Europe. The disease, which would eventually be named *Lyme borreliosis*, was probably first noted in Sweden in 1908. At that time, erythema chronicum migrans (recently simplified to **erythema migrans [EM]**) was described as a rash with expanding margins. In EM, red circles ring a white, hard center of the rash, resulting in a “bull’s eye” appearance ([Fig. 40.5](#)).

The next several decades saw more cases of EM in Europe. The first report of a similar phenomenon in the United States was in 1970. In 1975, an outbreak of juvenile rheumatoid

arthritis occurred in Lyme and Old Lyme, Connecticut. The clustered outbreak of juvenile rheumatoid arthritis appeared suspicious, and thanks to some family members who thought that an infectious agent might be involved, an investigation was initiated by health officials. It was Willy Burgdorfer’s accidental discovery of spirochetes in the blood of ticks recovered from the Old Lyme area that ultimately led to the description of Lyme disease. In 1984, the name *Borrelia burgdorferi* was proposed for these organisms. Lyme disease has a bimodal age distribution, with the first peak at 5 to 14 years of age and the second at 45 to 54 years of age.

Life cycle

Ixodes ticks typically have a 2-year life cycle, requiring blood meals to pass from the larval stage to the nymph stage and from the nymph stage to the adult stage. Nymphs and larvae feed primarily on the white-footed mouse, whereas adult ticks typically infest the white-tailed deer. This horizontal transmission ensures maintenance of the pathogen in the wild. Infected nymphs transmit the organism directly into the tissue of hosts (including humans) by regurgitating during feeding. Nymphs transmit the disease primarily in spring and summer. Their very small size poses the greatest threat because they are very hard to see.

Clinical manifestations

There are three stages associated with Lyme borreliosis: early localized, early disseminated, and late persistent. However, staging can be difficult because presentation varies by patient, and some may not exhibit all stages described in the typical illness. In about two thirds of infected patients, the early localized stage is characterized by a red papule that appears at the site of the tick bite within the first 30 days of infection. The papule, referred to as an *erythema migrans*, is the characteristic rash of Lyme borreliosis. It can expand to form erythematous concentric rings, with central clearing. Early

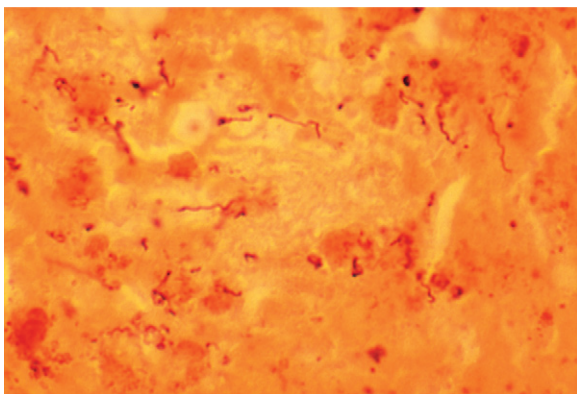


Fig. 40.4 Spirochetes of *Borrelia burgdorferi* (Dieterle silver stain preparation, $\times 1000$). (Courtesy Centers for Disease Control and Prevention, Atlanta, GA.)



Fig. 40.5 Patient with multiple erythema migrans lesions. (From Berger, B. W. [1989]. *Dermatologic manifestations of Lyme disease*, *Reviews of Infectious Diseases* 11, S1475.)

diagnosis and treatment can be very complicated in patients who do not exhibit EM because they may not even remember having been bitten by a tick.

The other symptoms associated with the early localized stage include nonspecific flulike symptoms, including malaise, lymphadenopathy, low-grade fever, headache, and neck stiffness. The second or early disseminated stage typically appears weeks to months after infection. During this stage, the most clinically significant symptoms are neurologic, such as Bell palsy, neurologic deficits, and meningitis. Other symptoms that may appear include oligoarthritis and carditis. Late persistent Lyme borreliosis is characterized by relapsing arthritis that can occur months to years after the initial symptoms. The joints most commonly affected are the knees, shoulders, and elbows. Untreated patients may have decreasingly severe attacks over time, until the symptoms eventually disappear.

Because the concentration of bacteria in the host remains low through the course of the disease, it is possible that many of the effects seen in Lyme borreliosis result from the host's immune response, including attraction of macrophages to synovial fluid and production of interleukin-1 by host monocytes. Lyme spirochetes are rarely isolated from the blood or other body fluids of infected patients. Apparently, the organism prefers solid tissue rather than fluid. The spirochete has a nonspecific adhesion that allows it to attach to the endothelial cells of blood vessels. This ability may enhance the migration of the organism from the bloodstream into the basement membrane, resulting in vascular damage that can lead to carditis, arthritis, and CNS disease. Treatment and management of Lyme borreliosis are guided by the patient's clinical presentation, stage of the disease, and the presence of any allergies.

Rickettsia infection

General characteristics

Rickettsia spp. are nonmotile, gram-negative bacilli, about 0.8 to 2.0 $\mu\text{m} \times 0.3$ to 0.5 μm in size; they are arthropod-borne,

obligate intracellular bacteria that can grow only in the cytoplasm of host cells. They spend at least part of their life cycle in an arthropod. Arthropod hosts generally serve as both reservoirs (transovarial transmission) and vectors (transmission between mammalian hosts) for members of the genus *Rickettsia*. Members of the genus have been grown in cell lines and embryonated eggs. However, because of their infectious nature, any attempt to cultivate them in clinical laboratories without proper equipment and training is not recommended. The rickettsiae are divided into two main groups according to the types of clinical infections they produce. The spotted fever group contains about a dozen species including *R. rickettsii*, *R. parkeri*, *R. conorii*, and *R. africae*; the typhus group consists of *R. prowazekii* and *R. typhi*. A third transitional group contains the human pathogens *R. akari*, *R. australis*, and *R. felis*.

Spotted fever group

Rocky Mountain spotted fever (RMSF), caused by *R. rickettsii*, is the most severe of the rickettsial infections and the most common in the United States. Humans acquire the infection by tick bites. *Dermacentor variabilis* in the southeastern United States and *D. andersoni* in the western part of the country are the vectors of *R. rickettsii*. Rodents and ticks serve as reservoirs. Incidence and case fatality rates of RMSF in the United States have been recorded since the 1920s (Fig. 40.6). Because of the difficulty and inability to differentiate between spotted fever group *Rickettsia* species using commonly available serologic tests, cases of RMSF have been reported under the category called spotted fever rickettsiosis (SFR). The category includes cases of RMSF, *Rickettsia parkeri* rickettsiosis, Pacific Coast tick fever (*Rickettsia* species 364D), and rickettsialpox (*R. akari*). According to the CDC, the number of SFR cases has increased in the past two decades, reaching a peak of 6248 in 2017. However, cases reported in 2018 and 2019 were slightly lower.

The bacteria preferentially infect the endothelial cells, where they replicate primarily in the cytoplasm of the host cell. Rickettsiae spread hematogenously throughout the host and

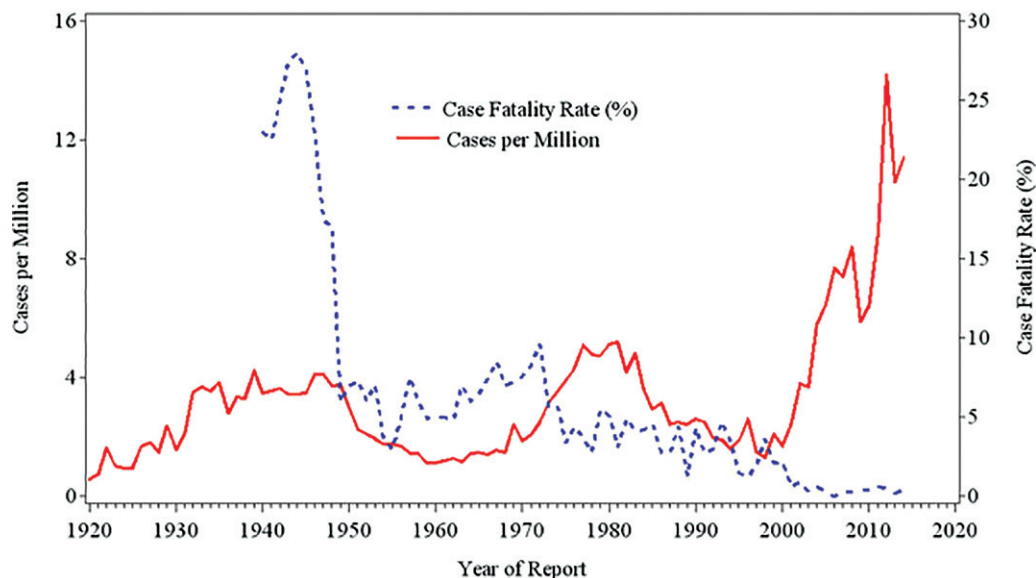


Fig. 40.6 Incidence and case fatality rate of Rocky Mountain spotted fever in the United States, 1920 to 2020. (Courtesy Centers for Disease Control and Prevention Atlanta, GA.)

induce vasculitis in internal organs, including the brain, heart, lungs, and kidneys. After an incubation period of approximately 7 days, the patient presents with flulike symptoms that include fever, headache, myalgia, nausea, vomiting, and rash. The rash begins as erythematous patches on the ankles and wrists during the first week of symptoms and then extends to the palms of the hands and soles of the feet. However, it normally does not affect the face. The maculopapular patches eventually consolidate into larger areas of ecchymoses. Other symptoms include hypotension and DIC. With rapid diagnosis and appropriate therapy, the mortality rate is 3% to 6%. The mortality rate without treatment can be 20% or higher.

Boutonneuse fever (BF), also known as *Mediterranean spotted fever*, is caused by *R. conorii* and occurs primarily in France, Spain, and Italy. *R. conorii* also causes Kenya tick typhus, South African tick fever, and Indian tick typhus. The brown dog tick, *Rhipicephalus sanguineus*, is the primary vector of *R. conorii* worldwide. Like the agent for RMSF, reservoirs include ticks and dogs.

BF clinically resembles RMSF because it causes a rash that commonly involves the palms of the hands and the soles of the feet. Unlike RMSF, the rash may also involve the face, and infection may be marked by a pathognomonic symptom known as **tache noire**, which is a small, 2- to 5-mm, black-centered ulcer that appears at the bite site. Other symptoms associated with BF include lymphadenopathy. BF is typically considered much milder than RMSF because the case fatality rate, even without treatment, is estimated at 3%, but severe complications with neurologic involvement occur in about 6% to 10% of patients.

Rickettsialpox, caused by *R. akari*, occurs in Korea and Ukraine as well as in the eastern United States. The disease was first reported in the United States in an apartment in the borough of Queens, New York, in 1946. Outbreaks have occurred in several large cities, including New York, Cleveland, and Philadelphia. The reservoir is the common house mouse, and the vector is the mouse mite. Infections occur in crowded urban areas where there are rodents and their mites. Rickettsialpox is similar to RMSF but milder. After an incubation period of about 10 days, a papule forms at the site of inoculation. The papule progresses to a pustule and then to an indurated eschar. Additional symptoms include headache, nausea, and chills. Unlike RMSF, the rash of rickettsialpox appears on the face, trunk, and extremities and does not involve the palms of the hands or soles of the feet. Rickettsialpox symptoms resolve without medical attention.

Typhus group

Murine typhus

Murine (endemic) typhus is caused by *Rickettsia typhi*. The arthropod vector is the oriental rat flea, and the rat is the reservoir. The cat flea can also serve as a reservoir. Because this flea infests a large number of domestic animals, it may be an important factor in the persistence of infection in urban areas. The rickettsiae also survive in nature, to a lesser extent, by transovarial transmission.

In the 1940s, approximately 5000 cases of endemic typhus were reported annually in the United States. Although it is no longer a reportable disease in the United States, fewer than 100 cases are reported annually. The disease essentially occurs only in southern Texas and southern California.

Worldwide, it continues to be a problem in areas where rats and their fleas are present in urban settings. As with RMSF, the clinical course of murine typhus includes fever, headache, and rash. Unlike RMSF, however, rash appears in only about 50% of patients with murine typhus. When the rash is present, it is usually on the trunk and extremities. Patients often have an eschar at the site of inoculation. Complications are rare, and recovery usually occurs without incident.

Louseborne typhus

Louseborne (epidemic) typhus is caused by *R. prowazekii*. Vectors of this disease include the human louse, squirrel flea, and squirrel louse. The reservoirs are primarily humans and flying squirrels located in the eastern United States. Unlike vectors of other rickettsiae, the louse often dies as a result of its rickettsial infection.

Louseborne typhus is still commonly found in areas of Africa and Central and South America, in areas where overcrowding and poor sanitation promote the presence of body lice. As demonstrated during World War II, epidemic typhus can occur even in developed countries when sanitation is disrupted. More than 20,000 cases of louseborne typhus were documented during the 1980s, with the vast majority originating in Africa.

Lice are infected with *R. prowazekii* when feeding on infected humans. The organisms invade the cells lining the gut of the louse and spill into the lumen of the gut when infected cells lyse. When the louse feeds on another human, it defecates, and the infected feces are scratched into the skin. Disease progression resembles that of RMSF, including a rash involving the palms of the hands and soles of the feet. Unlike RMSF, the face may also be affected by the rash.

Mortality rates in treated patients are very low; however, the mortality rates for untreated patients can approach 40%. **Brill-Zinsser disease**, or recrudescent typhus, is a reactivation of louseborne typhus. The bacteria can lie dormant in the lymph tissue of the human host for several years after the primary infection. Recrudescent typhus is milder than louseborne typhus, and death is rare. Patients with latent infections are an important reservoir for the organism.

Scrub typhus

Orientia tsutsugamushi (scrub typhus) is phylogenetically distinct from the rickettsiae. Many species of chigger mites in the family Trombiculidae serve as vectors for scrub typhus. Several mammalian hosts serve as reservoirs, with rodents being the principal reservoirs in Asia. Scrub typhus is common in southeast and central Asia and in the oriental region of the world. Scrub typhus is associated with rural areas where human risk is elevated when conducting nonmechanized agricultural practices of field sanitation or harvest. Annually nearly 1 million new cases are reported worldwide.

Chigger larvae are the only life stage that can acquire and transmit scrub typhus. *O. tsutsugamushi* is maintained in the chigger population through transstadial and transovarial transmission. Infection among the chigger population also occurs when uninfected chiggers cofeed on a host being parasitized by infected chiggers. Chigger larvae will infest rodent nests or other mammalian hosts. Unlike other rickettsia vectors, chiggers do not take a bloodmeal; they use their saliva to dissolve tissue, creating a stylostome. The stylostome, similar to a drinking straw, is the reason that bites have a pustule center.

O. tsutsugamushi is maintained in the salivary glands of the chigger and is transmitted to the host during stylostome development and subsequent feeding. Once in the mammalian host, *O. tsutsugamushi* begins to attack myelocytes and eventually endothelial cells near the stylostome. The pathogen survives the human immune response by interacting with host cells, causing them to create a clathrin-coated vesicle that transports *O. tsutsugamushi* to the cytoplasm, where it can begin attacking new cells and multiplying.

Humans are considered an incidental host but can serve as a reservoir to uninfected chigger populations. The chigger feeds on the host for 2 to 4 days and detaches. The stylostome leaves a pustule that is often itchy. Scrub typhus has symptoms similar to other rickettsial infections, which include fever, chills, body aches, enlarged lymph nodes, rash, and an eschar at the sight of the bite. If left untreated, patients can develop organ failure and bleeding, which can be fatal.

Anaplasmataceae infection

Case study

A 65-year-old male patient was experiencing fever, headache, myalgia, and anorexia for 5 days. When he went to his personal health care provider, his temperature was 38.3°C (101°F), reference range 35.6°C (96°F) to 37.5°C (99.5°F); his blood pressure was 128/60 mm Hg, reference range 90 to 119/60 to 79 mm Hg; and he was dehydrated. There was no sign of rash or lymphadenopathy. Serial blood culture results and routine serology tests were negative. The patient received doxycycline intravenously for 3 days. He then received doxycycline orally. His fever resolved after 6 hours of therapy. The following are the laboratory test results:

- WBCs: $2.5 \times 10^9/\text{L}$ (reference range 4.5 to $11 \times 10^9/\text{L}$)
- Platelets: $57,000/\mu\text{L}$ (reference range 150 to 400)
- Hematocrit: 41.1% (reference range 40% to 54%)
- Hemoglobin: 11.6 g/dL (reference range 14 to 18 g/dL)
- Aspartate aminotransferase: 167 U/L (reference range 6 to 34 U/L)
- Creatine phosphokinase: 403 U/L (reference range 20 to 200 IU/L)
- Alkaline phosphatase: 173 IU/L (reference range 44 to 121 IU/L)
- Total bilirubin: 1.7 mg/dL (reference range 0.1 to 1.2 mg/dL)

From Taylor J. P., et al. (1988). Serological evidence of possible human infection with *Ehrlichia* in Texas. *The Journal of Infectious Diseases*, 158, 217.

Causes

Members of the genera *Ehrlichia* and *Anaplasma* are the most important pathogens in the family Anaplasmataceae. *Ehrlichiosis* was first noted to cause lethal infection in dogs in the 1930s. Postmortem examination revealed rickettsial-like inclusions in the monocytes. The bacteria were named *Rickettsia canis*. They differ from other rickettsiae in that they multiply in the phagosomes of host leukocytes and not in the cytoplasm of endothelial cells. In addition, they do not appear to be transmitted transovarially in ticks; however, transstadial transmission is known to occur. Because of these differences, they were reclassified into the new genus *Ehrlichia* in 1945. They were eventually named *Ehrlichia chaffeensis*. Ehrlichiae, like chlamydiae, possess an infective form known as the *elementary body*,

which replicates in the phagosome. As the bacteria divide, they develop **morulae** (mulberry-like bodies; see Fig. 24.10). As the host cell ruptures, the morulae break into many individual elementary bodies that continue the infective cycle.

Epidemiology

Ehrlichiosis became reportable to the CDC in 1999. It is a seasonal disease, with the majority of cases occurring during the summer months of June and July. *E. chaffeensis* causes **human monocytic ehrlichiosis (HME)**. The infection occurs worldwide. In the United States, most cases are found in the southeastern and southcentral states as well as in the mid-Atlantic states. Fewer than 2000 cases have been definitively or presumptively identified since the disease description in 1986; however, cases may be underreported. Natural hosts of the organism include dogs, deer, and humans, with the lone star tick (*Amblyomma americanum*) being the primary vector.

Anaplasma phagocytophilum, formerly known as *Ehrlichia phagocytophilum*, causes a disease now referred to as **human granulocytic anaplasmosis (HGA)**. In 1994, molecular amplification and DNA sequencing showed that the causative agent of HGA was distinct from *E. chaffeensis*. From 2015 to 2019, roughly 3500 to 5700 cases were reported annually; like HME, diseases are probably underreported. Deer, rodents, horses, cattle, and humans are natural hosts. Tick vectors include *Ixodes scapularis* and *I. pacificus*.

Clinical manifestations

HME and HGA cases are similar clinically, and many patients may experience asymptomatic infection. The organisms have an incubation period of 5 to 10 days. Disease is clinically variable, but most patients have a moderately severe febrile illness. The most frequent manifestations are malaise (94%), fever (92%), myalgia (77%), and headache (75%). A few patients have arthralgia or involvement of the gastrointestinal tract (nausea, vomiting, diarrhea), respiratory tract (cough, pulmonary infiltrates, acute respiratory distress syndrome), liver, or CNS. Rash is observed in about 6% of patients. As many as 60% of pediatric patients infected by *E. chaffeensis* have a rash, although adults rarely experience a rash. Patients may also have evidence of leukopenia, thrombocytopenia, and elevated liver enzyme levels. Patients can experience severe complications, including illness similar to toxic shock-like syndrome, CNS involvement, and adult respiratory distress syndrome. Mortality rates are approximately 2% to 3%, but case severity may be increased in patients with impaired splenic function.

Case check 40.3

Diagnosis of ehrlichiosis and anaplasmosis is complicated because patients may not remember or know that they were bitten by an arthropod vector, and many of them present with non-specific symptoms. In addition, many of these causative agents are difficult or impossible to grow in culture and therefore may be detected only by using serology or other assays, which may be difficult to interpret. As a result, many of the illnesses in this category may be misdiagnosed, and population data, including prevalence and incidence of these diseases, may be grossly underestimated.

Trypanosomiasis

Cause

Trypanosomiasis is caused by flagellate parasites in the genus *Trypanosoma*. Trypanosomiasis is notable for causing Chagas disease (*Trypanosoma cruzi*) in South, Central, and North America and African trypanosomiasis, or sleeping sickness (*Trypanosoma brucei*). Kissing bugs (*Triatomine* spp.) are the vectors of *T. cruzi* and tsetse flies (*Glossina* spp.) the vector of *T. brucei*. The World Health Organization reported 750 cases of African trypanosomiasis in 2021, a 95% reduction compared to 2001. However, the World Health Organization estimates that 6 to 7 million people are currently infected with *T. cruzi*.

Life cycle

Vectors develop trypanosomiasis when they take a blood meal from an infected animal host. *Trypanosoma* multiply in the gut of the vector, eventually reaching the intestinal tract in kissing bugs or the salivary glands in tsetse flies. *T. cruzi* is passed to the host through the feces of kissing bugs. Kissing bugs feed on a host through a piercing sucking mouthpart similar to other hemipterans. While feeding, the kissing bug becomes engorged with blood and will excrete waste on the host. After feeding, the bite site becomes irritated, causing the host to scratch. Infection occurs when the feces come in contact with the bite site. *T. brucei* is passed to the host through the saliva of tsetse flies. A tsetse fly uses a stylet-like appendage to pierce the host's skin and draw blood. It then uses its spongelike mouthparts to soak up the blood. While feeding, the fly will excrete infected saliva into the host's wound, causing infection.

Clinical manifestations

African trypanosomiasis has two stages. Initially this disease manifests like many others discussed in this chapter with fever, headaches, enlarged lymph nodes, and joint pain. Eventually the parasites cross the blood-brain barrier and infect the CNS. This stage is known as the *neurologic* stage. Symptoms include confusion, sensory disturbances, lack of coordination, and changes in behavior. Disturbance of the sleep cycle is a hallmark of this disease.

The acute phase of Chagas disease has many of the same symptoms as those discussed in the first stage of African trypanosomiasis, with the addition of nausea, diarrhea, vomiting, loss of appetite, and enlargement of the liver or spleen. In certain individuals, eyelid swelling will occur if the bite and infection occurred near the eyes. These symptoms will pass. If left untreated, Chagas progresses into the chronic phase over a period of a couple decades. Symptoms can include stomach pain, constipation from an enlarged colon, difficulty swallowing caused by an enlarged esophagus, irregular heartbeat, heart failure, and sudden cardiac arrest.

glands and is transmitted by biting to other animals. Humans can also acquire infection by inhalation, which can be the case in bat caves. Other infections have been linked to transplanted tissue such as the cornea.

After the bite, viruses replicate locally for several weeks to months. However, the virus preferentially infects nerve cells. Viruses move along the peripheral nerves to the spinal cord. From here, the virus moves rapidly to the brain. Invasion of the brain produces encephalitis. Using afferent nerves, the virus moves to highly innervated tissue such as the scalp, salivary glands, retina, cornea, and nasal mucosa. The prodromal stage of rabies includes nonspecific symptoms like pain and paresthesia at the bite site, fatigue, and anorexia. After a period of 2 to 10 days, the neurologic signs begin: disorientation, hallucinations, hyperexcitability, difficulty swallowing, and the classic rabies symptom, hydrophobia. Less than 20% of patients exhibit paralysis. There is no specific antiviral therapy, and the disease is nearly always fatal.

Diagnosis is made based on history of animal contact, although in many cases no such history is recalled by the patient or family members. Skin sample taken from the nape of the neck along the hairline and saliva are recommended for viral detection. The direct fluorescent antibody test is the standard antigen detection method for tissue. Reverse transcriptase polymerase chain reaction is performed on saliva or tissue. Virus-specific antibodies can be detected in serum and CSF. The disease is sometimes diagnosed postmortem using tissue taken from the CNS.

In 2019, first encountered in Wuhan, China, severe acute respiratory syndrome–coronavirus-2 (SARS-CoV-2) was identified as the cause of a coronavirus-associated acute respiratory disease outbreak. The disease, now known as Coronavirus Disease 2019 (COVID-19), rapidly spread worldwide and was declared a pandemic by the World Health Organization in March 2020. The CDC reported the first person in the United States to die from COVID-19 on February 20, 2020. By July 2022, the World Health Organization reported that the COVID-19 pandemic caused over 550,000,000 confirmed cases and over 6,000,000 deaths. For the same period, the CDC reported over 87,000,000 U.S. cases with over 1,000,000 deaths. Researchers believe that SARS-CoV-2 has zoonotic origins, possibly emerging from a bat-borne or pangolin-borne virus because of its close genetic similarity to these coronaviruses.

A worldwide outbreak of monkeypox began in 2022 and was declared a Public Health Emergency of International Concern by the World Health Organization in July 2022. Before the outbreak, most cases were linked to international travel or imported animals. This previously rare disease was first described in monkey colonies used for research. However, the actual source of the virus remains unknown. African rodents may transmit the disease to humans. The first case of human monkeypox was described in 1970.

Chapter 29 in this book discuss the following viral zoonotic agents: Lake Victoria Marburg virus; Zaire, Reston, Sudan, and Tai Forest Ebola viruses; Rift Valley fever virus; henipaviruses; SARS-associated coronavirus; West Nile virus; and HPAI virus. Hepatitis E virus (HEV) is transmitted via the fecal-oral route. Cattle, rats, sheep, monkeys, goats, and especially pigs are vulnerable to HEV infection. In Nepal, most outbreaks have occurred after monsoon rains and flooding, fecal contamination of well water, and untreated raw sewage entering city water supplies.

Viral zoonotic diseases

Rabies, caused by the rhabdovirus, is a classic zoonosis. It is typically transmitted by the bite of infected animals, predominantly mammals, such as bats, cats, dogs, foxes, raccoons, and skunks. The virus infects the CNS of these animals, triggering aggressive behavior. The virus also inhabits the salivary

Emerging zoonoses

In recent years, the significance of zoonotic infections has continued to grow in prominence because of the emergence of several large-scale outbreaks in humans and animals. Although not all-inclusive, these have included the 2003 SARS, the low pathogenic avian influenza A virus subtype H7N2, the 2004 highly pathogenic avian influenza (HPAI) A virus subtype H5N1, the 2009 influenza A virus subtype H1N1 (swine flu), the 2010 *Escherichia coli* 0104:H4, COVID-19, and the 2022 monkeypox outbreak outbreaks.

The worldwide economic losses attributed to zoonotic diseases during the past decade were estimated at over \$200 billion before the ongoing COVID-19 pandemic. The social and economic impact of the pandemic on global gross domestic product growth is massive. In the United States alone, the total economic cost of the SARS-CoV-2 pandemic was estimated at trillions of dollars with lost jobs, service industries shattered, and education compromised. In addition, the emergence of novel zoonotic diseases has placed an undue burden on the medical industry because of the intrinsic difficulties associated with diagnosis, care, and treatment of patients.

The number and frequency of zoonotic disease outbreaks are increasing, which has sparked international interest and has resulted in a number of workshops, committees, and scientific groups sponsored by prestigious organizations, including the CDC, the U.S. Agency for International Development, the U.S. Department of Agriculture, the U.S. Department of Defense–Global Emerging Infections Surveillance and Response System, the World Health Organization, the Food and Agriculture Organization of the United Nations, the Office International des Epizooties (World Health Organization for Animal Health), and the Pan American Health Organization. The purpose of these organizations is to determine the root cause of these increases, identify strategies to minimize their economic and public health impact, and identify potential emerging zoonotic disease threats.

Although a number of factors have attributed to the increase in emerging and reemerging zoonoses, explosive population growth appears to be the most significant. According to statistics from the United Nations, the world population has more than doubled in the past 50 years (from 2.5 billion to 6.5 billion) and continues to increase at a rate of 1.7% per year. This increase has led to changes in land use, including deforestation of farmland and pastures, urbanization, and use of raw materials, all of which force humans and domestic animals to move into previously unpopulated areas and wild animals to change their migration routes and share their habitat with humans. Population growth also increases the demand for animal protein, forcing poultry, livestock, and animal products to be transported from rural areas to more populous areas and increases international trade of these commodities. The net result of all of these changes is increased interaction and microbial exchange among humans, domestic animals, and wild animals. It also provides a mechanism for the global spread of novel agents as they jump from species to species.

It is widely accepted that the key to reducing the impact of emerging zoonoses is global surveillance. However, just as the costs attributed to zoonotic outbreaks are staggering, so

are those associated with establishing and maintaining the required surveillance network. It is estimated that an investment of \$800 million per year would be required to support the required global surveillance and early response capabilities, including infrastructure building, capacity building, and research and development. In addition to these costs, there are two other significant roadblocks to implementation—garnering international cooperation and convincing resource-rich countries to provide developing nations with the required monetary resources so efforts can be focused in areas with the highest potential for emerging zoonotic infections.

In recent years, much emphasis has been placed on preparedness for influenza A virus pandemics. This has largely occurred because of the ability of influenza A virus to mutate and reassort with other influenza viruses, including those from other host species; the potential for the creation of a novel strain; and the efficiency of respiratory virus transmission. These abilities were highlighted by the 1918 influenza A virus subtype H1N1 (Spanish flu) pandemic, which killed between 50 million and 150 million people (3% of the world's population at the time). This explains the amount of concern generated by the recent emergence of two novel strains: the HPAI A virus subtype H5N1 (avian flu) in poultry and the 2009 influenza A virus subtype H1N1 (swine flu). Fortunately, both viruses lacked the key components necessary to make them as devastating as the 1918 strain. Swine flu could be transmitted very efficiently as demonstrated by the pandemic it caused, but it had a low mortality rate. However, avian flu has a very high mortality rate but currently lacks the ability for efficient person-to-person transmission. On the other hand, another respiratory virus, SARS-CoV-2 (COVID-19) was proven to be highly infectious and easily transmitted from person to person, and although most individuals are asymptomatic or have mild symptoms, those with certain medical conditions often progress to severe, life-threatening, and fatal illness.

Avian and swine flu as well as SARS are examples of **emerging zoonoses**. The term implies the discovery of a new agent, discovery of a known agent that has moved to a new geographic location, discovery of a known pathogen that has become drug resistant, or some other modification. These emerging zoonoses can cause human susceptibility to novel agents that jump from animal populations into human populations. By definition, some of the zoonotic agents already discussed fall into the category of emerging pathogens, including *B. burgdorferi*, *E. chaffeensis*, *A. phagocytophilum*, *B. henselae*, and *T. cruzi*. Emerging zoonotic diseases are of concern and highlight the necessity for continued pandemic surveillance, planning, and antiviral stockpiling.

Case check 40.4

Outbreaks of novel zoonotic diseases are becoming increasingly common, and the economic losses associated with them are staggering. Countermeasures to prevent them or reduce their impact are complicated and require a significant capital investment. However, based on recent data, the estimated cost associated with developing prevention capability is several orders of magnitude less than the economic toll attributed to outbreak responses over the past decade.

POINTS TO REMEMBER

- Testing for zoonotic infections represent a significant percentage of the workload in a clinical microbiology laboratory.
- Animal exposure and insect bites are important clues about possible causes of a patient's infection.
- Zoonotic agents can be bacterial or viral as well as parasitic or mycotic.
- Not all zoonotic infections are contagious. In several zoonotic infections, the pathogens spread efficiently to humans but do not spread easily from person to person. Many zoonotic infections are arthropod-borne.
- For the past 30 years, emerging infections have been recorded, including the discovery of previously unknown pathogens. Of these, 75% have a zoonotic origin.

LEARNING ASSESSMENT QUESTIONS

- Erythema migrans (EM) is a rash associated with which zoonotic infection?
- Late persistent Lyme borreliosis is usually characterized by which type of clinical manifestation?
- Which two animals are most often associated with human cases of pasteurellosis?
- Which two preexisting medical conditions increase the risk of *Capnocytophaga canimorsus* infection in humans?
- Which organism is most likely associated with cat scratch disease (CSD)? What is another organism that can also cause CSD?
- Which toxins are responsible for life-threatening edema in pulmonary anthrax?
- Which four species of brucellae are pathogenic to humans, and what are the normal hosts for these organisms?
- What are the differences between icteric and anicteric leptospirosis?
- Which zoonotic agents are responsible for causing human granulocytic anaplasmosis (HGA) and human monocytic ehrlichiosis (HME)? What underlying medical issue may increase the severity of these diseases?
- Which species are included in the typhus group of the genus *Rickettsia*?
- Describe the pathogenesis of Rocky Mountain spotted fever.
- Define emerging zoonotic disease. What is the worldwide cost attributed to zoonotic outbreaks in the past decade?
- Which of the following is not a clinical manifestation of pasteurellosis?
 - Cellulitis
 - Corneal ulcers
 - Stomach ulcers
 - Pericarditis
- Which genera of tick includes the primary vectors of *Borrelia burgdorferi*?
 - Dermacentor*
 - Amblyomma*
 - Rhipicephalus*
 - Ixodes*
- In which zoonotic illness are humans the primary reservoir?
 - African trypanosomiasis
 - Pasteurellosis
 - Cat scratch disease
 - Plague
- Which zoonoses have an eschar associated with them?
 - Lyme borreliosis and rickettsial disease
 - Rickettsia disease and anthrax
 - Cat scratch fever and leptospirosis
 - Anthrax and trypanosomiasis
- Which of the following is *not* a manifestation of anthrax?
 - Cutaneous
 - Pulmonary
 - Intestinal
 - Subcutaneous
- Which of the following is *not* a vector or vehicle of transmission for tularemia?
 - Animal bites
 - Aerosols
 - Contaminated blood
 - Contaminated soil
- What is another name for Weils syndrome?
 - Rocky Mountain spotted fever
 - Icteric leptospirosis
 - Cutaneous anthrax
 - African trypanosomiasis
- Which typhus is associated with chiggers?
 - Murine typhus
 - Epidemic typhus
 - Scrub typhus
 - Both a and c
- Which typhus is associated with Brill-Zinsser disease?
 - Epidemic typhus
 - Scrub typhus
 - Murine typhus
 - Both b and c
- Which of the following is a vector of African trypanosomiasis?
 - Kissing bug
 - Tick
 - Tsetse fly
 - Flea

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Ocular infections

Gail Reid

CHAPTER OUTLINE

General concepts related to ocular infections, 1002

- Ocular structures, 1002
- Ocular microbial biota, 1002
- Host protective mechanisms, 1003
- Virulence factors of ocular pathogenic organisms, 1003

Infections of the conjunctivae, 1004

- Bacteria, 1005
- Viruses, 1007
- Fungi, 1008
- Parasites, 1008

Infections of the eyelids, 1008

- Bacteria, 1008
- Viruses, 1008

Infections of the cornea, 1009

- Bacteria, 1009
- Viruses, 1010
- Fungi, 1010
- Parasites, 1011

Infections of the sclera and episclera, 1012

Infections of the orbit, 1012

Infections of the lacrimal apparatus, 1013

Infections of the intraocular chambers, 1014

- Bacteria, 1014
- Fungi, 1014
- Parasites, 1014

Retinitis, 1014

Biofilm-centered ocular infections, 1014

Laboratory diagnosis of ocular infections, 1016

- Specimen collection, 1016
- Direct microscopic examination, 1016
- Culture and identification, 1017
- Molecular techniques, 1019
- Special culture techniques, 1020

Bibliography, 1022

OBJECTIVES

After reading and studying this chapter, you should be able to:

1. Describe the most important ocular structures and their functions.
2. Discuss the role of normal biota in protecting ocular structures.
3. Describe the most common ocular infections and their causative agents.
4. Describe the pathogenesis of the various eye pathogens presented in this chapter.
5. Associate the sources and risk factors for particular pathogens causing ocular infections.
6. Explain the virulence mechanisms associated with ocular pathogenic organisms.
7. Justify the laboratory procedures for recovery and identification of ocular pathogens.
8. Compare the administration methods for antimicrobials used in the treatment of ocular infections with those used in the treatment of other types of infections.

KEY TERMS

Blepharitis	Keratitis
Canaliculitis	Keratoconjunctivitis
Conjunctivitis	Orbital cellulitis
Dacryoadenitis	Preseptal cellulitis
Dacryocystitis	Scleritis
Endophthalmitis	Stye

Case in point

A healthy 20-year-old female patient with no history of ocular disease started wearing disposable contact lenses 3 months prior. She replaced them every 7 to 10 days. The patient developed blurred vision, pain, photophobia, and redness in her right eye during the 12th week. An examination revealed conjunctival edema and four small corneal ulcers with a green, mucopurulent discharge. Smears for Gram and Giemsa stains were prepared from the discharge. Cultures were performed from corneal scrapings and from the contact lens and solution.

Issues to consider

After reading the patient's case history, consider:

- The role that the technique for handling, insertion, and removal of a contact lens may have in establishing ocular infections
- The need for rapid diagnosis in this case
- Common pathogens involved in this type of infection

Any organism capable of gaining entrance to ocular structures can cause disease. Ocular infections range from relatively mild, self-limiting **conjunctivitis** (inflammation of the conjunctiva) and **blepharitis** (inflammation of the eyelids) to the more severe and sight-threatening conditions **keratitis** (inflammation of the cornea) and **endophthalmitis** (inflammation of the inside lining and cavities of the eyes). The surrounding soft tissues, sclera, lacrimal system, and bony orbit are also subject to microbial invasion. Bacteria, fungi, viruses, and protozoa all play prominent roles in the pathogenesis of ocular disease. The selection of specific microbiological procedures is dependent on the severity of symptoms, site of infection, and likely pathogen.

General concepts related to ocular infections

Ocular structures

Fig. 41.1 depicts the most important ocular structures. The visual system is composed of the eyeball (globe), muscles, fat, nerves, orbital bones, and neural pathways that carry electric impulses that are converted into vision. This system does not actually “see”; instead, it acts as a receptor for sensory light stimuli that are translated into neural impulses by the retina and then processed by the occipital lobe of the brain. In a sense, the eye is a frontal extension of the brain.

The conjunctiva is a mucous membrane covering the inside lining of the eyelids and white covering of the eyeballs (sclera). It is similar to the membranes in the mouth and nose, and it constitutes the front line of defense against invading organisms. The tears that keep the conjunctiva moist contain many enzymes and other factors (e.g., immunoglobulin A [IgA], phospholipase A₂, complement proteins, lysozyme, lactoferrin, mucins) that protect the conjunctiva from microbial invasion.

The eyelids are thin elastic layers or folds of tissue that help protect the structures of the orbit. They are the thinnest skin covering in the body. The blinking action of the eyelids and conjunctivae help keep the cornea and sclera lubricated and sweeps away debris and potential pathogens. The eyelashes act as a filtering and monitoring system that alerts the brain to potentially harmful agents.

The cornea is considered the window of the eye. Its function is similar to that of a crystal on a wristwatch. The internal ocular structures can be viewed through the cornea. The function of the curved corneal surface is to refract, collect, and focus light onto the retina. A layer of tears (the tear film) blankets the cornea to provide optical clarity, lubrication, and nutrition. Many free nerve endings are located in the corneal epithelium. When there is a break in or an injury to the

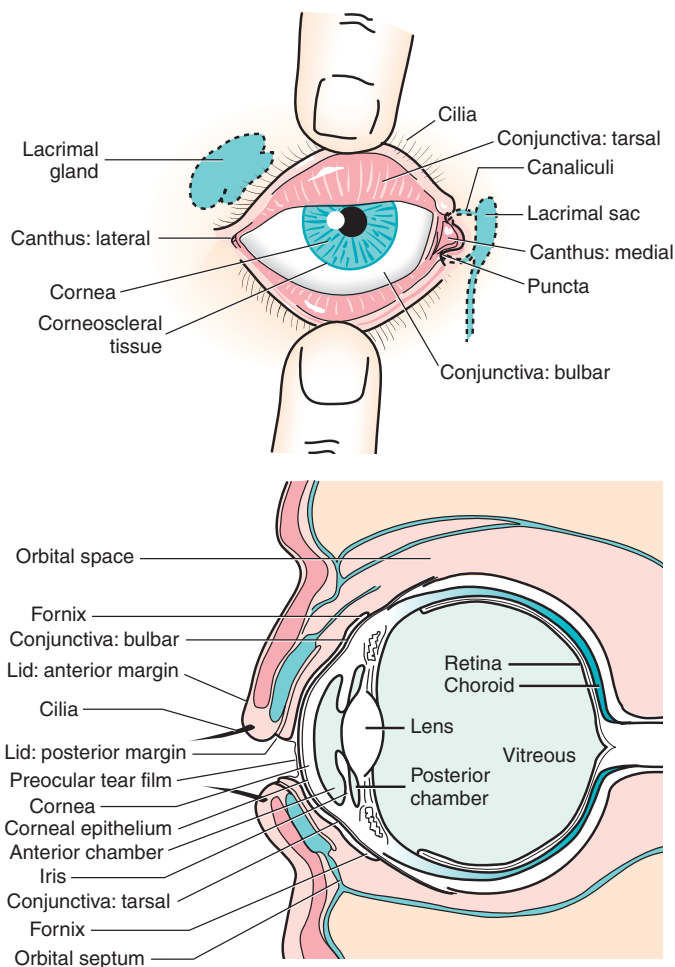


Fig. 41.1 Common ocular structures. (Modified from Jones, D. B., et al. (1981). *Laboratory diagnosis of ocular infections*. In J. A. Washington (Ed.), Cumitech 13. Washington, DC: American Society for Microbiology.)

epithelium, the patient usually complains of considerable pain. Infections and injury to the cornea are considered true ocular emergencies.

Ocular microbial biota

Coagulase-negative staphylococci and *Corynebacterium* spp. make up 80% to 90% of the indigenous microbiota recovered from noninflamed eyes. However, depending on the age of the individual, season, location, and underlying conditions, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, and other potential pathogens may be recovered from noninfected eyes (Table 41.1). The presence of a resident biota on the conjunctivae and eyelids can be a protective mechanism, inhibiting invasion and colonization by more harmful organisms. The normal resident conjunctival and eyelid microbiota change with age.

The distinction between indigenous microbiota and ocular pathogens can be blurred. Coagulase-negative staphylococci, *Cutibacterium acnes*, and *S. aureus* are responsible for many intraocular and corneal infections. The presence of biomaterials and the use of steroids, antimicrobials, and contact lenses can predispose ocular tissues to infection with indigenous microbiota. In the United States, an estimated 38

Table 41.1 Ocular resident biota from noninflamed eyes

Organisms ^a	Incidence (%)
Coagulase-negative staphylococci ^b	34–94
<i>Cutibacterium acnes</i> ^b	40–86
<i>Corynebacterium</i> spp. ^b	3–83
<i>Staphylococcus aureus</i>	0–30
<i>Haemophilus influenzae</i>	0–25
<i>Micrococcus</i> spp.	2–22
<i>Streptococcus pneumoniae</i>	0–5
Viridans group streptococci	0–12
Gram-negative rods (<i>Proteus</i> spp., <i>Escherichia coli</i> , <i>Klebsiella pneumoniae</i> , <i>Enterobacter</i> spp.)	0–5
<i>Moraxella</i> spp. (including <i>Moraxella catarrhalis</i>)	0–3
<i>Bacillus</i> spp. (<i>Bacillus cereus</i> , <i>Bacillus subtilis</i>)	0–4
<i>Neisseria</i> spp. (<i>Neisseria sicca</i> , <i>Neisseria flavescens</i>)	0–7
Fungi (any saprophyte, depends on locale)	0–24
Anaerobic biota other than <i>Cutibacterium acnes</i> , <i>Peptostreptococcus</i> spp., <i>Bacteroides</i> spp., <i>Clostridium</i> spp.	1–5
β-Hemolytic streptococci, including <i>Streptococcus pyogenes</i>	0–3
Sterile	9–47

^aSource is usually the conjunctivae and eyelids; the organism isolated depends on the age of the patient, geographic locale, season, previous and current therapy, and underlying condition (e.g., diabetes, epithelial disease). Most common isolates usually reflect that of the surrounding tissue.

^bCan also cause mild to severe disease in immunocompromised patients.

million individuals wear contact lenses. Although contact lenses pose a substantial risk for eye infections, such infections are relatively uncommon in individuals wearing them. Daily disposable contact lenses pose a lower risk of infection than reusable lenses. The presence of an unapparent biofilm (see [Chapter 31](#)) in the storage case has been cited as a source of contact lens contamination. In addition, the antimicrobial agents used in storage solutions might not be effective against some strains of *Pseudomonas aeruginosa* or *Acanthamoeba* spp.

Case check 41.1

The patient in the Case in Point presented with symptoms suggestive of conjunctivitis and keratitis. She is at increased risk for this type of infection because of the use of contact lenses. It is important that individuals use proper hand hygiene while inserting and removing contact lenses.

Organisms may be recovered from the conjunctival sac and eyelids without causing infection. Quantitative ocular cultures have been used to establish a threshold to differentiate between infection and colonization. A threshold is set for each organism, and infection is established when organisms reach or exceed the threshold number. It is important

to remember that once the epithelium of the conjunctiva or cornea is compromised, any organism gaining entrance can result in disease, and the presence of any amount of organisms in what should be a sterile site is indicative of infection.

Host protective mechanisms

Because of their location, external ocular structures and surfaces, such as the conjunctivae and cornea, are frequently challenged by a variety of microorganisms. Whether an infection or damage ensues depends on the structure, immune status, and response of the host; integrity of the underlying tissues; and pathogenesis of the invading organism. Protection of ocular structures is partly supported by a defense system that includes local and systemic, specific and nonspecific, and humoral and cellular mechanisms that join to prevent microbial colonization or invasion. Their protection is further supported by an anatomic arrangement that leaves the inner ocular structures well sequestered. The intact epithelia of the eyelid, conjunctiva, and cornea provide a protective barrier against invasion by most microbes.

Tears contain high concentrations of IgA, lysozyme, and lactoferrin. All three have antimicrobial properties. IgA coats bacteria, aids in complement fixation and phagocytosis, and can inhibit colonization by pathogens. Lysozyme attacks bacterial cell walls, primarily those of gram-positive bacteria, by splitting bonds in the peptidoglycan layer. Lactoferrin inhibits the growth of bacteria by competing for and binding to iron. Secretory phospholipase A₂ has activity against gram-positive bacteria by binding to the bacterial cell surface and killing the cell by lipolytic enzymatic activity. The blinking action of the eyelids and flow of tears also protect the eye from infection by removing bacteria and debris from the ocular surface. Cooler ocular surface temperatures can inhibit the growth of many microorganisms.

Risk factors associated with ocular infections include age, sex, race, socioeconomic status, behavior, geographic location, occupation, and underlying disease. Acute bacterial and viral conjunctivitis occur more frequently in childhood, whereas chronic conjunctivitis and varicella-zoster virus (VZV) conjunctivitis occur most frequently in older adults. *S. aureus* and *Candida albicans* are usually recovered from patients with keratitis in cooler climates; *P. aeruginosa* and filamentous fungi are the main pathogens recovered from patients in warmer climates. Fungal or traumatic keratitis has been diagnosed in farmers and agricultural workers. *S. pneumoniae* and *Moraxella* spp. are recovered from people with alcoholism and those who are homeless. Women are more likely than men to have trachoma, a serious eye infection caused by *Chlamydia trachomatis*, whereas chlamydial inclusion conjunctivitis is most often identified in men.

Virulence factors of ocular pathogenic organisms

Several microbes can penetrate the intact epithelium of the conjunctiva or cornea, including *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *S. pneumoniae*, *Listeria monocytogenes*, and *Corynebacterium diphtheriae*. For most other microbes to enter and establish disease, a break must occur in the protective epithelial barrier. Once the intact epithelium is breached (e.g., by trauma, by insertion or removal of a contact lens), an

intrusion by pathogenic and saprophytic organisms can occur. Many ocular pathogens possess adhesions and enzymes that aid in adherence, multiplication, and dissemination in ocular tissues. *S. aureus*, *P. aeruginosa*, and coagulase-negative staphylococci may persist in ocular tissue or biomaterials in a biofilm, which protects them from host defenses, antimicrobial agents, and eradication. Penetrating eye trauma caused by a soil-contaminated object can result in *Bacillus cereus* infection that frequently results in complete loss of the eye within 48 hours.

When an infection has started in one layer of the eye, spread to adjacent layers and tissues can occur rapidly. Dissemination of the infection within ocular structures can result in devastating and permanent damage to the functional integrity of the eye. Box 41.1 lists organisms recovered from ocular infections. Any organism that can gain entrance to the

internal structures of the eye is capable of causing infection. The list is long and varied. Organisms considered contaminants or colonizers are the most frequent pathogens. Many systemic illnesses, such as tuberculosis, syphilis, diabetes, hypertension, and acquired immunodeficiency syndrome (AIDS), can also have ocular symptoms. The organism most likely to be encountered depends on the season, climate, age of the patient, and underlying disease.

Infections of the conjunctivae

Conjunctivitis is the most common ocular complaint. It includes all age groups and occurs worldwide. An estimated 6 million people in the United States develop conjunctivitis annually,

BOX 41.1 Microorganisms associated with ocular infectious disease

Bacteria

Gram-negative (aerobic)

Alcaligenes spp.
Acinetobacter spp.
Aggregatibacter actinomycetemcomitans
Achromobacter xylosoxidans
Aeromonas hydrophila
Bartonella henselae
Borrelia tularensis
Brucella spp.
Capnocytophaga spp.
Eikenella corrodens
Enterobacterales
Flavobacterium spp.
Francisella tularensis
Haemophilus aegyptius
Haemophilus influenzae
Haemophilus parainfluenzae
Kingella spp.
Neisseria gonorrhoeae
Neisseria meningitidis
Moraxella catarrhalis
Moraxella lacunata
Pseudomonas aeruginosa

Gram-negative (anaerobic)

Bacteroides spp.
Fusobacterium spp.
Prevotella spp.

Gram-positive (aerobic)

Bacillus cereus
Bacillus spp.
 β -Hemolytic streptococci (A, B, C, F, G)
Corynebacterium spp.
Enterococcus faecalis
Listeria monocytogenes
Micrococcus spp.
Staphylococcus aureus
Staphylococcus epidermidis and other coagulase-negative staphylococci
Streptococcus pneumoniae
Viridans group streptococci

Gram-positive (anaerobic)

Actinomyces israelii
Actinomyces spp.

Clostridium spp.
Peptostreptococcus spp.
Cutibacterium acnes
Propionibacterium propionicus

Aerobic actinomycetes

Nocardia spp.
Streptomyces spp.

Chlamydia and similar organisms

Chlamydia trachomatis
Chlamydia pneumoniae
Chlamydia psittaci
Coxiella burnetii

Mycobacteria

Mycobacterium abscessus
Mycobacterium avium complex
Mycobacterium chelonae
Mycobacterium fortuitum
Mycobacterium gordonae
Mycobacterium leprae
Mycobacterium mucogenicum
Mycobacterium nonchromogenicum
Mycobacterium triviale
Mycobacterium tuberculosis

Parasites

Acanthamoeba spp.
Ascaris lumbricoides
Leishmania spp.
Loa loa
Microsporidia spp.
Onchocerca volvulus
Phthirus pubis
Taenia solium
Toxocara canis and *Toxocara cati*
Toxoplasma gondii
Trichinella spiralis
Trypanosoma spp.
Vahlkampfi spp.
Wuchereria bancrofti

Viruses

Adenoviruses
Coxsackieviruses
Cytomegalovirus

Enteroviruses
Epstein-Barr virus
Herpes simplex virus types 1 and 2
Human herpesviruses 6, 7, and 8
Human immunodeficiency virus
Human papillomavirus
Influenza virus
Measles virus
Molluscum contagiosum virus
Mumps virus
Vaccinia virus
Varicella-zoster virus

Fungi

Molds

Acremonium spp.
Alternaria spp.
Aspergillus spp.
Bipolaris spp.
Blastomyces dermatitidis
Coccidioides immitis
Cladosporium spp.
Colletotrichum spp.
Curvularia spp.
Drechslera spp.
Exophiala jeanselmei
Fusarium spp. (*Fusarium oxysporum* and *Fusarium solani* most common)
Histoplasma capsulatum
Paecilomyces spp.
Penicillium spp.
Phialophora spp.
Mucor spp.
Rhizopus spp.
Scedosporium apiospermum
Sporothrix schenckii
Volutella spp.

Yeasts

Candida albicans
Candida spp., other than *C. albicans*
Cryptococcus neoformans
Rhodococcus spp.
Torulopsis glabrata

and 1% of all primary care visits are related to this condition. Symptoms may include itching, tearing, a foreign-body sensation, discharge (purulent or watery), and hyperemia, or increased blood flow resulting in “red eye.” Red eye constitutes more than 50% of office visits to ophthalmologists and is the most common ocular reason for microbiological evaluation.

Conjunctivitis may be acute, hyperacute, subacute, or chronic. Viruses are the most common cause of infectious conjunctivitis, and bacteria are the second most common cause. Fungal and parasitic infections are seen less frequently (Box 41.2). Uncomplicated acute viral conjunctivitis is generally self-limiting and does not require treatment. Infections by *N. gonorrhoeae*, *C. trachomatis*, and other bacteria should be treated. Treatment may be topical, except for gonococcal and chlamydial infection, which should be treated with systemic antimicrobials. Corneal involvement may follow conjunctivitis and compromise vision. Chronic infections are more difficult to manage and result in long-term ocular morbidity and compromised vision. Up to 40% of the U.S. population experiences allergic conjunctivitis, but the majority of these individuals do not seek treatment.

Bacteria

Acute bacterial conjunctivitis

A statistically significant positive correlation was reported between conjunctivitis and weekly sunshine duration and weekly average temperatures at a U.S. hospital. This indicates

that more cases of conjunctivitis occur in warmer weather or that patients are more likely to seek treatment in warmer weather. Other studies have reported that cases of bacterial conjunctivitis peak during December to April, whereas more cases of viral conjunctivitis occur in the summer. Staphylococci, in particular *S. aureus*, are the most frequently isolated pathogens, followed by *S. pneumoniae* and *H. influenzae*. In children with acute conjunctivitis, *H. influenzae*, *S. pneumoniae*, and *Moraxella catarrhalis* are the most frequently isolated organisms. *N. gonorrhoeae* (Fig. 41.2) and *C. trachomatis* cause a hyperacute conjunctivitis that produces large

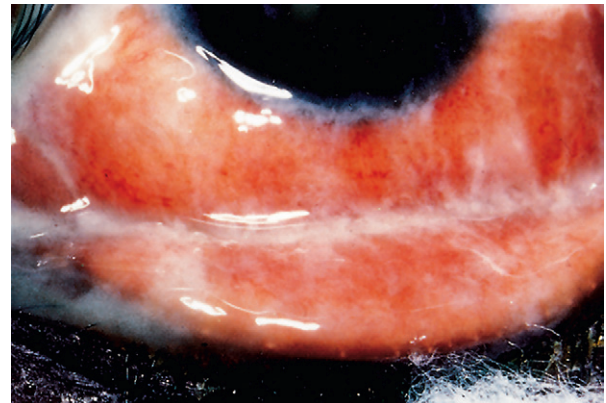


Fig. 41.2 Gonococcal conjunctivitis. Note the copious discharge in response to invasion by *Neisseria gonorrhoeae*.

BOX 41.2 Microorganisms associated with conjunctivitis

Bacteria

Gram-negative

Acinetobacter spp.
Bartonella henselae
 Enterobacterales (e.g., *Escherichia coli*, *Proteus mirabilis*,
Shigella spp.)
Francisella tularensis
Haemophilus influenzae
Haemophilus ducreyi
Moraxella catarrhalis
Moraxella lacunata
Neisseria gonorrhoeae
Neisseria meningitidis
Pseudomonas aeruginosa
Yersinia enterocolitica

Gram-positive

β -Hemolytic streptococci (A, B, C, G)
Corynebacterium diphtheriae
Corynebacterium spp.
Listeria monocytogenes
Mycobacterium tuberculosis
Staphylococcus aureus
Staphylococcus epidermidis
Streptococcus pneumoniae
 Viridans group streptococci

Chlamydia

Chlamydia pneumoniae

Chlamydia psittaci

Chlamydia trachomatis

Viruses

Adenoviruses
 Human coronaviruses
 Herpesviruses (1–8)
 Influenza virus
 Paramyxoviruses (measles, mumps, and Newcastle disease viruses)
 Picornaviruses (echovirus, enterovirus, coxsackievirus, poliovirus)
 Poxviruses (molluscum contagiosum virus and vaccinia virus)

Fungi (rare)

Candida spp.
Coccidioides immitis
Rhinosporidium seeberi
Sporothrix schenckii

Parasites (rare)

Ascaris lumbricoides
 Fly larvae (*Oestrus ovis*, myiasis)
Loa loa
 Microsporidia
Onchocerca volvulus
Pthirus pubis
Taenia solium
Trichinella spiralis
Toxocara canis
Wuchereria bancrofti

amounts of exudate. Infants can acquire *N. gonorrhoeae* as they pass through an infected birth canal. Symptoms may appear within 5 to 7 days after exposure to the pathogen. It is believed that adults acquire gonococcal conjunctivitis through self-inoculation from a genital infection. Meningococcal conjunctivitis may result from contiguous spread from the respiratory tract.

Chronic bacterial conjunctivitis

The causative agents in chronic conjunctivitis, defined as cases lasting longer than 4 weeks, are less clear. The microorganisms that have been isolated most frequently include *S. aureus*, *Moraxella lacunata*, and enteric bacteria. Coagulase-negative staphylococci and *C. acnes* have been seen less frequently. Chronic conjunctivitis may result from interplay between the organism and an aggressive ocular immune response. Bacterial conjunctivitis can also be caused by instillation of contaminated cosmetics or medications. The organisms encountered in such infections are *S. aureus*, *Staphylococcus epidermidis*, *Corynebacterium* spp., *P. aeruginosa*, and *Proteus mirabilis*. Allergic and chemical conjunctivitis can sometimes be confused with microbial infections.

Laboratory diagnosis

Laboratory tests can assist in differentiating acute, allergic, and chronic conjunctivitis. Conjunctival scrapings are collected using a Kimura spatula, blade, or sterile swab and plated directly onto culture media and slides. Routine stains (e.g., Gram, Giemsa) and culture should reveal the causative agent in most acute cases. Smears and culture specimens should be collected in all cases of purulent, membranous, or pseudomembranous conjunctivitis. Laboratory workup is mandatory for all cases of neonatal and infant conjunctivitis. Culture and examination of smears may be of less value in establishing the causative agent in chronic conjunctivitis.

Treatment

Many bacterial conjunctivitis cases are self-limiting and resolve without treatment. Antimicrobial agents can reduce symptoms and prevent secondary infections. Initial, empiric topical therapy is often sufficient. Therapy is then adjusted based on the organism isolated and antimicrobial susceptibility testing.

Chlamydial ocular infections

C. trachomatis causes a myriad of ocular infections, including neonatal conjunctivitis, inclusion conjunctivitis (Fig. 41.3A), and trachoma. Currently, 20 serotypes or serovars of *C. trachomatis* have been described, and specific serovars are associated with certain clinical manifestations. Trachoma, which is as old as recorded history, is usually caused by serovars A, B, Ba, or C, whereas serovars D to K are usually associated with oculogenital inclusion conjunctivitis.

Neonatal conjunctivitis becomes apparent within 8 to 10 days after birth. The infant is infected while traveling through the infected birth canal. *C. trachomatis* inclusion conjunctivitis can lead to pharyngitis, otitis media, and interstitial pneumonitis in the newborn. Chronic conjunctivitis with cornea involvement is a consequence of untreated or inadequately treated inclusion conjunctivitis. Trachoma is a leading cause of blindness worldwide. Regional variation exists in prevalence and severity. Worldwide, 1.9 million people have trachoma, and 200 million people live at risk of the disease. It is endemic in some of the poorest countries in Africa, Central and South America, Asia, Australia, and the Middle East. The initial conjunctivitis is follicular conjunctivitis, followed by repeated infections, which lead to scarring and blindness. Nasolacrimal duct obstruction and dacryocystitis (infection of the lacrimal sac caused by obstruction, usually seen in children) are complications that follow trachoma. Chronic conjunctivitis leading to scarring and chronic keratitis may follow trachoma and inclusion conjunctivitis. Blindness is irreversible.

The prevalence rates for ocular chlamydial infection parallel those of genital disease. In areas with high rates of sexually transmitted infections (STIs), ocular disease rates are also high. Populations with the highest incidence of disease include neonates and sexually active adolescents and adults. The incidence can range from 20% to 90%, depending on the age group. In areas with low rates of STIs, ocular chlamydial infection rates also are low.

Chlamydia pneumoniae was originally isolated from the conjunctiva of a child. Most adults will have antibodies against this organism. Acute ocular infections with this organism are rare. It has been isolated most often in lower respiratory tract infections.

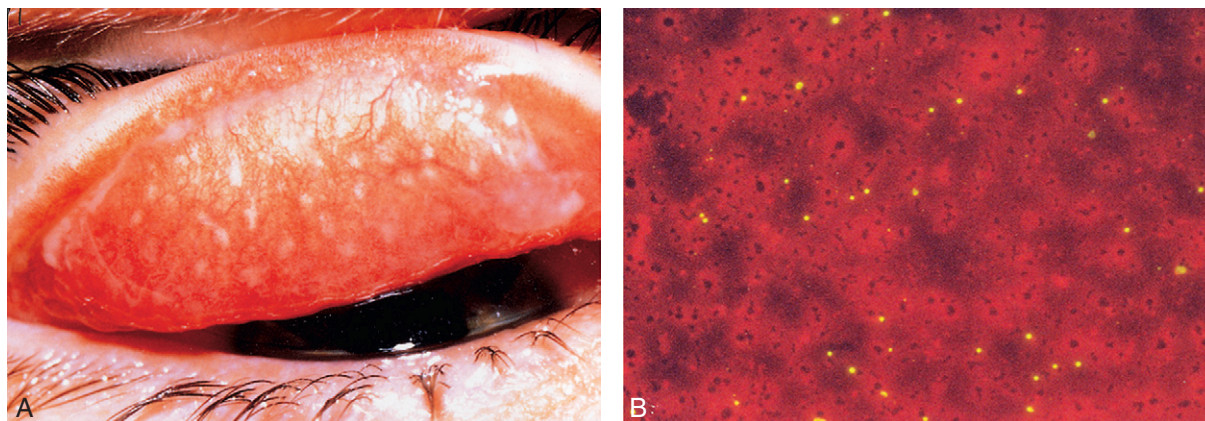


Fig. 41.3 A, White spots on the conjunctiva represent pockets of *Chlamydia* organisms in tissue. B, Immunofluorescence stain of scrapings from neonatal conjunctivitis, confirming the presence of chlamydial elementary bodies.

Laboratory diagnosis

Direct detection of the chlamydial inclusions in scrapings or on impression cytology membranes is generally considered a sensitive method for confirming ocular *Chlamydia* infections (see Fig. 41.3B). *Chlamydia* inclusions can be seen using Giemsa-stained smears and fluorescent-labeled monoclonal antibodies. *C. trachomatis* and *C. pneumoniae* can be isolated in tissue culture using McCoy or human epithelial type 2 (HEp-2) cells. However, nonculture detection methods, such as polymerase chain reaction (PCR) assay, direct fluorescent antibody assay, and enzyme immunoassay (EIA), are more frequently used to detect ocular *Chlamydia* infections. Nucleic acid amplification tests (NAATs) are considered the methods of choice because of their high sensitivity and specificity.

Treatment

Although erythromycin is administered prophylactically to neonates to prevent eye infections caused by *C. trachomatis*, its efficacy is not clear. However, ocular prophylaxis with erythromycin prevents gonococcal ophthalmia, and it should be administered. Topical treatment alone is inadequate to treat eye infections caused by *C. trachomatis*. Current recommendations by the Centers for Disease Control and Prevention (CDC) for the treatment of ocular chlamydial infection are as follows:

- Adults: azithromycin, 1 g orally in a single dose; or doxycycline, 100 mg orally twice a day for 21 days
- Neonates: erythromycin, 50 mg/kg per day orally, in four daily doses for 14 days; or azithromycin, 20 mg/kg per day orally, one dose daily for 3 days

Viruses

Viral conjunctivitis is a commonly recognized ocular infectious disease. It may also present as an acute or chronic illness, ranging from a mild, self-limiting condition to a severe, destructive disease resulting in impaired vision. Corneal involvement often follows viral infection of the conjunctiva. Healthcare-associated transmission of adenoviruses can occur during ocular examinations because the virus can persist in optic solutions or ophthalmic equipment. Use of single-dose packages or sterile drops augmented with frequent hand-washing can reduce the risk of transmission to patients and health care providers. A tonometer, a device used to measure intraocular pressure, should be cleaned with alcohol, rinsed with sterile water, and allowed to dry. Patients exposed to contaminated body fluids, fomites, or vesicular fluids are at increased risk of infection with herpes simplex virus (HSV), VZV, or epidemic keratoconjunctivitis (EKC), an infection of the cornea and conjunctiva.

Acute viral conjunctivitis

Acute viral infections are attributed mainly to adenoviruses, herpesviruses, or enteroviruses. The adenoviruses are responsible for two distinct ocular viral syndromes, EKC and pharyngoconjunctival fever (PCF). EKC is contagious and associated with adenovirus types 8 and 19, but recovery of serovars 7, 9, 10, 11, 14, and 16 has also been documented. PCF is frequently caused by adenovirus type 3 and occasionally by serovars 1,

2, 4, 5, 6, 8, and 14. Both syndromes are self-limiting and have no specific treatment; PCF lasts about 10 days, and EKC lasts 3 to 4 weeks at most. EKC may be spread through direct contact, fomites, and, not uncommonly, health care providers. It is usually responsible for outbreaks in ophthalmic offices and clinics, school locker rooms, and college dormitories.

Acute hemorrhagic conjunctivitis (Fig. 41.4), or epidemic hemorrhagic conjunctivitis (EHC), is an acute, short-lived infection caused by enterovirus 70, coxsackievirus A24, and, on rare occasions, adenovirus type 11. The onset of symptoms usually occurs within 8 to 48 hours of exposure, with patients complaining of pain, sensitivity to light, copious tears, and subconjunctival hemorrhages. It is self-limiting and has no outlined treatment program. Recovery occurs within 5 to 7 days. Because EHC is highly contagious and easily spread from person to person, patients should be isolated until the condition has resolved.

HSV blepharoconjunctivitis is responsible for most severe ocular viral infections. This disease usually occurs in young children. It is important to distinguish between HSV and adenovirus because HSV can be treated with specific ocular antiviral medications, whereas adenovirus infection cannot. More than 90% of cases of HSV blepharoconjunctivitis are caused by HSV-1, but HSV-2 has been isolated from infants and adults. VZV may also cause conjunctivitis. This condition is usually a manifestation of the systemic disease chickenpox or a complication of herpes zoster ophthalmicus. Cytomegalovirus (CMV) can cause conjunctivitis when it becomes disseminated or is transmitted through tears from another patient. Epstein-Barr virus (EBV) conjunctivitis may result as a complication of infectious mononucleosis. Chronic viral conjunctivitis may result from molluscum contagiosum or vaccinia, a complication of smallpox vaccination.

Laboratory diagnosis

Conjunctival scrapings may be stained with fluorescent-labeled monoclonal antibodies to confirm infection with an ocular viral pathogen. NAATs are also available for several of the viral agents and are the preferred method. The most common pathogens may be grown in cell culture and serotyped with monoclonal antibodies or neutralization tests.

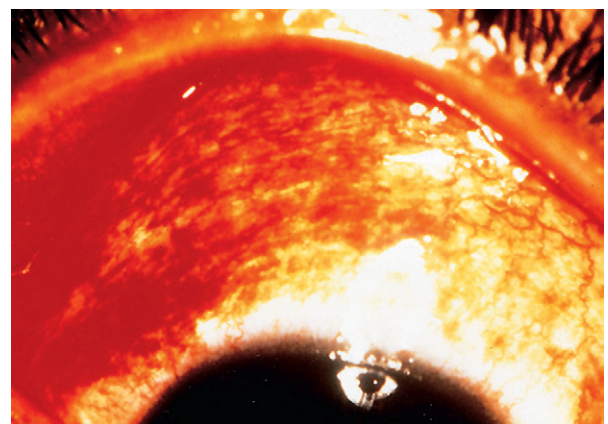


Fig. 41.4 Acute hemorrhagic conjunctivitis. The causative agent is usually enterovirus 70 or coxsackievirus A24. Other members of the enterovirus group may also be recovered. Note the heavy conjunctival hemorrhaging.

Treatment

Currently, there is no treatment for adenoviral and/or enteroviral ocular infections. Cold compresses may help alleviate some of the symptoms. Orally administered acyclovir/valacyclovir can be used to treat HSV and VZV infections.

Fungi

Although various fungi can be isolated from the uninflamed eye, fungal conjunctivitis is relatively rare. The organisms that have been recovered from fungal conjunctivitis are *Candida* spp. and *Sporothrix schenckii*. Systemic fungal infections caused by *Coccidioides immitis*, *Histoplasma capsulatum*, and *Cryptococcus neoformans* may extend to the conjunctival tissue and cause disease. To diagnose fungal conjunctivitis, the health care provider must have a high index of suspicion, and the laboratory must be alerted to ensure that the proper setup for recovery of these organisms is available. Laboratory diagnosis includes recovery on routine media and detection on Gram- and Giemsa-stained smears. Natamycin and amphotericin B ocular preparations are used to treat common infections.

Parasites

Parasitic infestation of the conjunctiva is generally rare. Ocular sites are occasionally a secondary complication of systemic parasitic worm infections. Onchocerciasis (river blindness), transmitted by the bite of the blackfly, is a leading cause of blindness in Africa and is in limited areas of South America and the Middle East. Ocular complications result from the discharge of large numbers of microfilariae by the adult female and their subsequent local invasion and tissue damage leading to keratitis from chronic infection. Recovery and diagnosis of ocular involvement depends on observing and removing the larvae (microfilariae) and confirming their presence through histologic staining, isolating the organisms from blood or tissues, or confirming their presence by serologic means. The recommended treatment for onchocerciasis is ivermectin, which kills the larvae and prevents them from causing damage but does not kill the adult worms. Ivermectin will need to be given every 6 months for the life span of the adult worms or as long as there is evidence of infection.

Infections of the eyelids

Blepharitis and conjunctivitis are not mutually exclusive. Conjunctivitis usually presents as blepharoconjunctivitis. Therefore any organism that causes conjunctivitis can affect the eyelids. However, each site has unique organisms and conditions. The skin covering the eyelids is the thinnest in the body. Any organisms capable of initiating skin infections can also cause blepharitis.

Bacteria

S. aureus and the coagulase-negative staphylococci are the bacteria most frequently isolated from the eyelid margins. Blepharitis involving these organisms is a low-grade inflammation associated with functional disease of the seborrheic glands (seborrheic blepharitis). In this mixed infection, dry

(staphylococcal) and greasy (seborrheic) scales are attached to the eyelashes, with various areas of ulcerations that cause the lashes to fall out. Antimicrobial agents are given to treat the staphylococcal disease. Because the scalp, eyebrows, and eyelids are all involved in seborrhiasis, all must be kept clean by using a medicated shampoo.

Four types of glands are located in the lids: the meibomian gland, the glands of Moll and Zeis, and accessory lacrimal glands. Acute infection of the glands of Zeis or Moll with staphylococci results in an external hordeolum (stye). A **stye** is an abscess with pus formation in the lumen of the affected gland. Hot soaks assist in the continuous drainage of the abscess. Erythromycin or tetracycline may be applied topically as supplementary therapy. An internal hordeolum caused by staphylococci is a little larger and affects the meibomian gland. Only rarely does the laboratory receive a request for culture of eyelid specimens. If such a request is received, it is to confirm the presence of staphylococci and determine whether therapy is adequate. Other microbial agents recovered from patients with blepharitis are listed in [Box 41.3](#). *Actinomyces* spp. may spread from the facial skin to the eyelids. *Mycobacterium* spp. that cause ocular infections are listed in [Box 41.1](#).

Viruses

Viral blepharitis can be caused by HSV-1, HSV-2, VZV, poxviruses, or papovaviruses. HSV infection usually occurs during early childhood. Vesicles appear on the eyelid margins and the skin around the eye. The vesicles break open and form crusted secondary lesions, which can then become superinfected by skin organisms. Direct detection with immunofluorescence or immunoperoxidase can be done by scraping the

BOX 41.3 Microorganisms associated with blepharitis

Bacteria

Common isolates

Staphylococcus aureus
Staphylococcus epidermidis and other coagulase-negative staphylococci
 Group A and other β -streptococci
Moraxella lacunata, *Moraxella* spp.

Rare isolates

Bacillus cereus
Haemophilus ducreyi
Clostridium spp.
Actinomyces spp.
Mycobacterium tuberculosis
Mycobacterium leprae

Fungi (rare)

Candida spp.
Cryptococcus neoformans
Blastomyces dermatitidis
 Dermatophytes (*Microsporum*, *Trichophyton*, *Epidermophyton* spp.)

Viruses

Herpes simplex virus types 1 and 2
 Varicella-zoster virus
 Molluscum contagiosum virus
 Vaccinia virus
 Papovavirus

base of a freshly opened vesicle. Vesicular fluids are collected for culture; 95% or more of cultures grow HSV within 72 hours or less.

When the face is involved during episodes of chickenpox (varicella), vesicles may appear on the upper or lower eyelid margins. Molluscum contagiosum is a wartlike lesion of the eyelid margins produced by a poxvirus. The lesion is waxy and pearly white, with an umbilicated center. Expression (squeezing) of the white center to allow blood into the lesion is usually adequate management. Vaccinia infection of the eyelids results from direct inoculation from a smallpox vaccination. Other viruses that produce ocular warts are members of the papovavirus family.

Infections of the cornea

Microbial keratitis is considered a true ocular emergency. Few organisms can invade the intact cornea. If the cornea epithelium is breached by trauma, contact lens insertion and removal, or surgery, organisms can enter and cause infections. Infection begins in the most superficial layer (the epithelium); if unchecked, infection advances through the Bowman zone, stroma, and the Descemet membrane to the endothelium (the innermost layer). Perforation of the Descemet membrane compromises vision and must be treated aggressively with antimicrobials and/or surgical intervention.

In the United States, contact lens use is considered the greatest risk factor for developing microbial keratitis. The incidence of keratitis related to extended-wear contact lenses is approximately 20 per 10,000 people, although the incidence for daily-wear contact lenses is about 1 per 10,000 people. A study in France found that the greatest risk factor for keratitis was using disinfecting solution for more than 3 months (odds ratio 1.94). Additional risk factors for microbial keratitis include trauma and refractive surgery and poor hygiene when handling lenses.

Bacteria

Geographic variations in the causes of bacterial and fungal ulcers are evident in the United States. *P. aeruginosa* is the isolate most frequently recovered from patients with contact

lens-associated keratitis. *S. aureus* is another frequently recovered pathogen (Fig. 41.5). There is also a seasonal distribution of causative agents. One study in Brooklyn, New York, found that in cases occurring in summer, 47.6% of culture-positive ulcers had *P. aeruginosa*. In the remaining seasons, *P. aeruginosa* was found in less than 13% of positive cultures. In this study, 39% of the patients with keratitis wore contact lenses. Fig. 41.6A shows the growth of *P. aeruginosa* from a contact lens. Fig. 41.6B lists trends in the types of bacteria recovered from contact lenses and solutions.

Mycobacterium chelonae, *Mycobacterium abscessus*, and other rapid-growing mycobacteria are being isolated with increasing frequency from patients with keratitis (Fig. 41.7). Risk factors include laser-assisted in situ keratomileusis (LASIK) eye surgery, trauma, and contact lens use. Reservoirs include contaminated instruments and/or soil and water. Less frequently encountered corneal isolates are listed in Box 41.4.

Laboratory diagnosis

Laboratory assistance with diagnosis is mandatory in all cases of suppurative keratitis or keratitis not responding to therapy. Ideal specimens for examination of smears and for culture are corneal scrapings, which are collected by an ophthalmologist. Material should be inoculated directly onto culture plates and slides and sent immediately to the laboratory. Scrapings for stains can provide the health care provider with an early indication of the offending organism and assist in the selection of appropriate therapy. The cornea may also be invaded via metastatic spread from the conjunctiva or systemic lesions; therefore other specimens might be useful in diagnosis.

Case check 41.2

The best specimen for the diagnosis of keratitis is a corneal scraping, as was submitted in the Case in Point. The green mucopurulent discharge is suggestive of *P. aeruginosa* conjunctivitis and/or blepharitis. To reduce the risk of permanent eye damage and vision loss, it is important to begin appropriate therapy immediately. This would involve empiric treatment with a broad-spectrum antimicrobial agent before complete culture results are obtained.

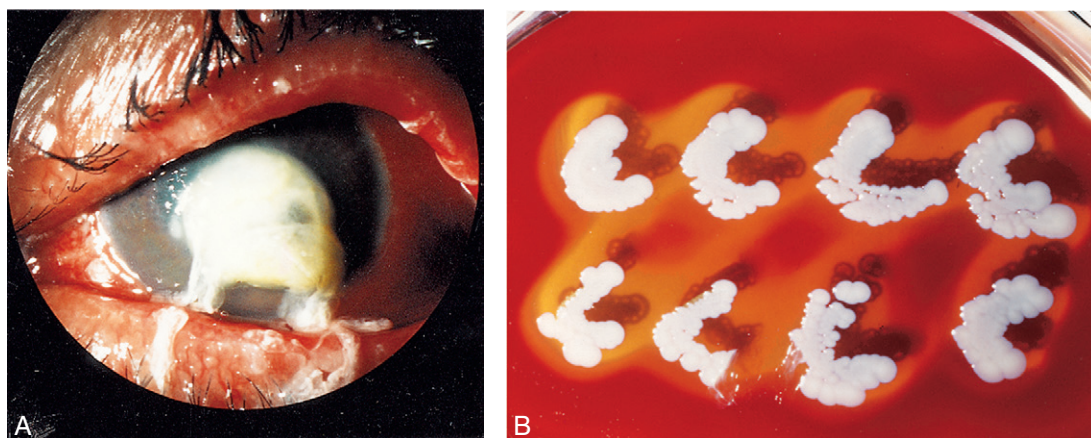
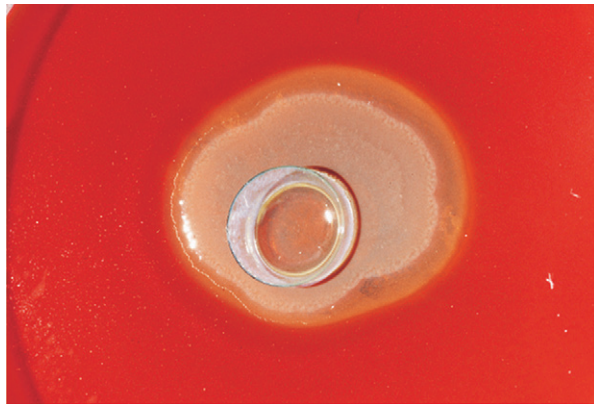
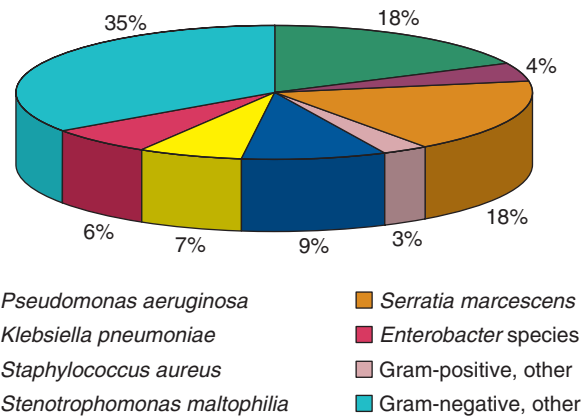


Fig. 41.5 A, Corneal melt caused by bacterial invasion. B, C streaks of *Staphylococcus aureus* from an infected cornea. The enzymes produced by some strains of *S. aureus* and *Pseudomonas aeruginosa* can liquefy the cornea within 48 hours.



A



B

Fig. 41.6 A, Growth of *Pseudomonas aeruginosa* from daily wear (soft) contact lens. The patient had an ulcerative keratitis. The corneal culture also grew *P. aeruginosa*. B, Recent trends in bacteria recovered from contact lenses and solutions.

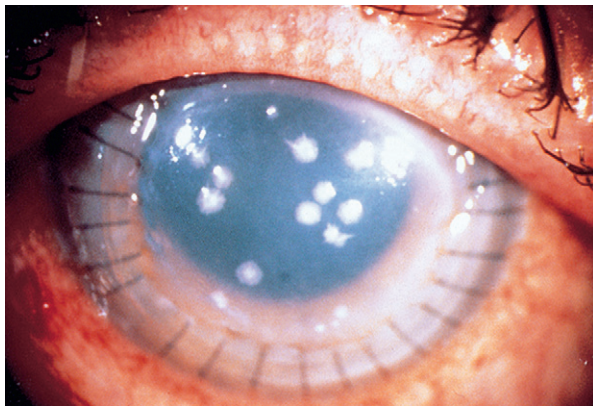


Fig. 41.7 Colonies of *Mycobacterium fortuitum* growing on infected corneal graft tissue.

BOX 41.4 Isolates

Mycobacterium abscessus
Mycobacterium asiaticum
Mycobacterium avium complex
Mycobacterium chelonae
Mycobacterium fortuitum
Mycobacterium gordonae
Mycobacterium mucogenicum
Mycobacterium nonchromogenicum
Mycobacterium szulgai
Mycobacterium triviale

Mycobacterium tuberculosis and *Mycobacterium leprae* are ocular pathogens and are usually an extension of systemic disease. Isolates are frequently recovered from patients who wear contact lenses, have a history of eye trauma from soil or water, or have undergone refractive surgery.

Treatment

Therapy is often selected on the basis of the results of smear examinations and/or chosen empirically for the most likely pathogen. Management includes topical antimicrobial drops or ointments supplemented with oral or systemic antimicrobials.

Viruses

HSV is a leading cause of infectious blindness and ocular morbidity in the United States and other countries.

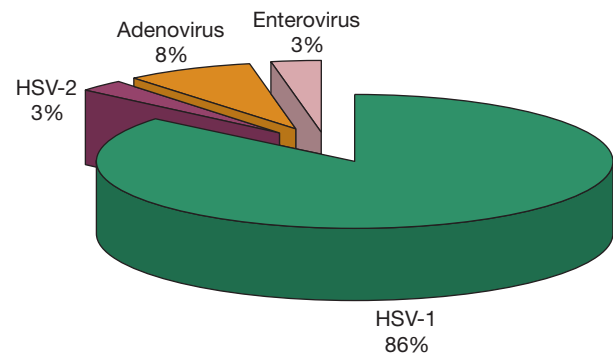


Fig. 41.8 Frequency of common keratitis viral isolates in southern Florida. HSV-1, Herpes simplex virus type 1; HSV-2, herpes simplex virus type 2.

Worldwide, approximately 1.5 million new cases of HSV keratitis occur each year, including 40,000 cases of visual impairment or blindness. Keratitis caused by HSV may result from direct inoculation or reactivation of latent virus in the trigeminal ganglion. Most of the ocular lesions are caused by HSV-1, but HSV-2 has been recovered from cases involving infants and adults. Fig. 41.8 shows the frequency of common keratitis viral isolates in cases in southern Florida. Scarring and blindness result from repeated outbreaks and surgical interventions. The lesions caused by these two are indistinguishable.

Laboratory diagnosis includes direct detection using monoclonal antibodies, DNA probes, PCR assays, and/or growth in cell culture. Treatment includes debridement, when possible; topical antivirals; and corneal transplantation. Other viral agents that cause keratitis are listed in Box 41.5.

Fungi

Fungal infections of the cornea are usually associated with opportunistic or saprophytic organisms. More than 70 genera have been documented as causative agents in fungal keratitis. Filamentous organisms, mainly *Fusarium*, *Aspergillus* spp., *Scedosporium apiospermum*, and *Paecilomyces*, are common pathogens in warm climates, whereas *Candida* spp. are common in cooler climates. Patients often have a history of

trauma involving soil or plant material and occurring most frequently in agricultural workers. Other high-risk factors include allergic conjunctivitis, eye surgery, treatment with broad-spectrum antimicrobial agents and corticosteroids, and contact lens use. In 2005 to 2006, the CDC reported an outbreak of at least 130 confirmed cases of *Fusarium* sp. keratitis associated with contaminated contact lens solution. All cases of fungal keratitis must be confirmed by the laboratory. Fungal keratitis agents are listed in Box 41.5.

Samples for culture must be collected from multiple scrapings of the involved, actively advancing edges of the ulcer. Biopsy is sometimes necessary to isolate the fungi. Giemsa and calcofluor white preparations from scrapings can confirm

BOX 41.5 Microorganisms associated with keratitis

Bacteria

Gram-negative isolates

Haemophilus influenzae
Moraxella spp.
Proteus mirabilis
Pseudomonas aeruginosa
Serratia marcescens

Gram-positive isolates

Corynebacterium spp.
Staphylococcus aureus
Staphylococcus epidermidis and other coagulase-negative staphylococci
Streptococcus pneumoniae
 Viridans group streptococci

Uncommon isolates

Nocardia spp.
Capnocytophaga spp.
Cutibacterium acnes
 Mycobacteria other than tubercle bacilli
Acanthamoeba and other free-living amoebae

Viruses

Adenoviruses
 Cytomegalovirus
 Enteroviruses
 Epstein-Barr virus
 Herpes simplex virus types 1 and 2
 Human immunodeficiency virus (tears and stroma)
 Measles virus
 Mumps virus
 Vaccinia virus
 Varicella-zoster virus

Fungi

Acremonium spp.
Alternaria spp.
Aspergillus spp.
Bipolaris
Candida spp. (*Candida parapsilosis* and *Candida tropicalis* most common)
Curvularia spp.
Fusarium spp. (*Fusarium solani* and *Fusarium oxysporum* most common)
Paecilomyces spp.
Phialophora spp.
Scedosporium boydii
Trichosporon beigelii

hyphal elements or budding yeasts. Topical antifungals include natamycin, nystatin, and imidazole. Topical preparations from systemic antifungals like voriconazole and amphotericin B may be used to manage fungal keratitis. Systemic antifungals may be needed for perforated ulcers. Corneal transplantation is often necessary to restore sight.

Parasites

Amoebae

Infection of the cornea by free-living amoebae (e.g., *Acanthamoeba* spp., *Naegleria* spp., and *Vahlkampfia* spp.) is a painful and sight-threatening consequence of injury or exposure to contaminated water, contact lenses, or soil. Wearing contact lenses while swimming in contaminated water or showering is a high risk factor for infection. In addition, topping off new solution with old, and not following proper protocol in handling, disinfecting, and storing contact lenses can lead to biofilm formation by bacteria, such as *P. aeruginosa*. This can provide a nutrient-rich environment for amoebae. *Acanthamoeba* spp. are the most frequent isolates. There have also been reports of contamination of contact lens solution. Amoebic keratitis is often mistaken for viral or fungal keratitis, and a diagnosis of amoebic keratitis is generally made when culture or NAAT results are negative for bacteria, fungi, and viruses. Confocal microscopy of corneal scrapings along with clinical findings had a reported sensitivity of 90.6% and a specificity of 100% for *Acanthamoeba* keratitis.

One method to cultivate the amoeba is to place a few drops of a heavy suspension of *Escherichia coli* or *Enterobacter aerogenes* in the middle of a petri dish containing nonnutrient media of 1.5% Difco agar made with Page's saline. The drop is spread by using a sterile bacteriologic loop. Ocular material is then added to the center of the plate. The bacteria serve as a nutrient source for *Acanthamoeba* spp. The amoebic trophozoites are identified by locating them at the end of the tracts they generate as they ingest the bacteria. Depending on the quality of the scrapings and infectious dose of the organism, *Acanthamoeba* trophozoites may be seen on agar within 48 hours. The polygonal, double-walled cysts may be detected on a direct smear with Giemsa or calcofluor white staining. Trophozoites are much less discernible on smears and are best seen in tracts on the agar (Fig. 41.9).

Another method is to inoculate scrapings into cell cultures. The amoebae cause a generalized destruction of the tissue culture cells. A third method of cultivation is to grow free-living amoebae in broth culture (axenic, free from other living organisms). The presence of *Acanthamoeba* spp. may also be confirmed by using molecular techniques.

Treatment of *Acanthamoeba* keratitis is difficult and includes debridement in early disease. No amoebicidal agents are currently approved. Patients are often treated with a combination of drugs, such as topical propamidine or hexamidine (0.1%) solution with biguanide chlorhexidine (0.02% to 0.2%) or polyhexamethylene biguanide (0.02% to 0.06% solution). Corneal transplantation is often necessary.

Microsporidia

Microsporidial keratitis, seen in immunocompromised patients, such as those with AIDS, is a disease that has appeared recently. Infections have also been seen in immunocompetent

contact lens wearers. Microsporidiosis is usually associated with contact with bees or other insects. The microsporidia are intracellular, spore-forming fungi. Because their morphology resembles protists, they are often discussed with the parasites. In humans, both ocular and nonocular diseases caused by these organisms have been reported. These pathogens are obligate intracellular parasites that infect ocular conjunctivae and corneal tissue. Their size ranges from 1 to 20 μm and is round or spherical (Fig. 41.10). The organism develops in two stages: the schizogenic phase and the sporulation, or sporogenic, phase. There are several pathogens in this group: *Encephalitozoon* spp., *Nosema oculorum*, and *Vittaforma corneae*.

Spores may be detected with hematoxylin and eosin, Gram, Giemsa, acid-fast, or calcofluor white stains. Because of their small size, it takes training and expertise to accurately identify microsporidia in tissue specimens. Some microscopists prefer the tissue Gram stains Brown-Brenn or Brown-Hopps or a silver stain (e.g., Warthin-Starry stain). Electron microscopy is usually required for taxonomic classification. Detection of ocular parasites is based on the following: (1) observing the organism, (2) confirming the presence of the organism with histologic or other staining methods; and (3) isolating the organism from blood, tissues, or body fluids. No uniform treatment is available, and treatment depends on the organism, the patient's underlying conditions (such as HIV/AIDS)

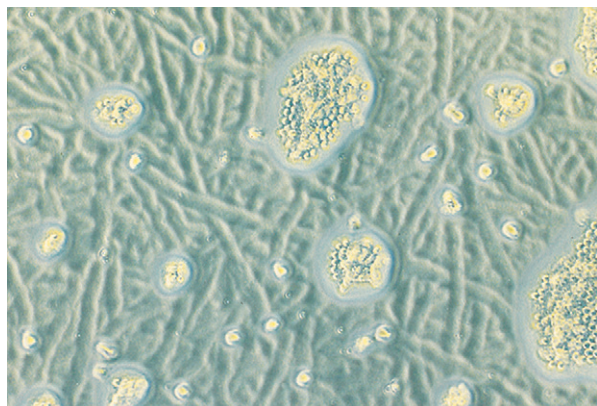


Fig. 41.9 Tracts of *Acanthamoeba* trophozoites. The meandering trophozoites are at the end of the tracts. The large clusters of organisms contain trophozoites and cysts (unstained).

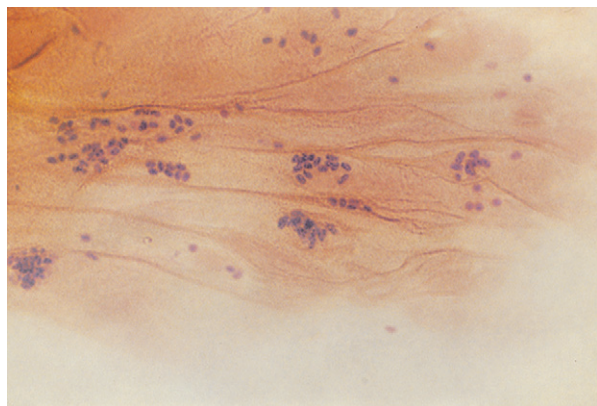


Fig. 41.10 Gram-stained smear revealing the oval cyst of *Microsporidia* spp. Organisms can also be detected with Giemsa, acid-fast, and calcofluor white stains.

and availability of an effective anti-infective. In addition, if there is disseminated disease, an extended course of oral albendazole is also recommended.

Infections of the sclera and episclera

The sclera is composed of tough collagen fibers, and few organisms can penetrate this strong protective coat. Infections are protracted, painful, and quite destructive. **Scleritis** is usually a local manifestation of systemic connective tissue disease (e.g., rheumatoid arthritis, systemic lupus erythematosus) or the result of contiguous spread of infection from adjacent ocular tissues. The presentation may be acute (pyogenic) or chronic (granulomatous). The episclera is the outermost layer of the sclera. Inflammation of this tissue is termed *episcleritis*. Episcleritis is more common than scleritis and has an undetermined cause in 70% of cases.

Laboratory diagnosis includes collection of tissue or discharge from the site and plating on routine and special media for common and infrequent isolates. Treatment is dependent on the organisms. Surgical intervention may be necessary to retard progression.

Infections of the orbit

Preseptal cellulitis is infection of the eyelids and soft tissues surrounding the orbit. It is a medical emergency in children. Infection can lead to blindness or death if not treated immediately and aggressively in all age groups. Predisposing factors include trauma, lower respiratory tract infections, impetigo, and herpetic diseases. *S. aureus*, *H. influenzae*, *S. pneumoniae*, and *S. pyogenes* are recovered in most preseptal cellulitis cases. Laboratory testing includes examination of direct Gram-stained smears and aerobic and anaerobic cultures. Broad-spectrum coverage for *S. aureus*, *H. influenzae*, *S. pneumoniae*, and *S. pyogenes*, which constitute 95% of the microorganisms recovered from cases of preseptal cellulitis, is recommended.

Infections of the orbit and associated structures may have a devastating effect on vision and ocular structural integrity. The close proximity of the orbit and related tissues to the paranasal sinuses, the absence of an effective drainage system from this sequestered area, and the unique structure of the eyelids predispose this area to invasion by various microorganisms. Infectious agents can gain entry into the orbital tissue through trauma or injury to the eyelids or orbit resulting from surgery, infections of the eyelids and adjacent skin, upper respiratory tract infections, and dental caries. Parasites may also invade the orbital tissue and cause considerable destruction. Orbital infections can also be extensions of bacterial or fungal sinus infections. Any organisms that initiate sinusitis can also cause **orbital cellulitis**. Various anaerobic organisms are isolated from samples from patients with orbital cellulitis associated with longstanding chronic sinusitis. Most of these infections are caused by bacteria, but saprophytic fungi may also be involved. Usually, both aerobic and anaerobic bacteria are isolated from these infections.

Chronic orbital cellulitis may be caused by *Mycobacterium* spp., *Nocardia* spp., and *Actinomyces* spp. Bacterial infections associated with orbital implants or prostheses present increasing threats to vision. The organisms recovered include

M. chelonae, *Nocardia* spp., *S. epidermidis*, *Capnocytophaga* spp., *Candida parapsilosis*, and *C. acnes*.

Trichinosis, caused by the nematode *Trichinella spiralis*, can invade the extraocular muscles and result in periorbital edema and pain on eye movement. It is the ophthalmologist who usually makes the diagnosis of trichinosis because invasion of the ocular muscles is generally one of the first signs of this disease. Other parasitic infections are rare.

Aspirates for smears and cultures are collected and placed onto slides by an ophthalmologist and sent immediately to the laboratory. Stains from tissues and/or aspirates can afford the health care provider an early indication of the offending organism and assist in the selection of appropriate therapy. Systemic therapy should be instituted as soon as appropriate culture samples (conjunctiva, nasal, and blood) are collected and should provide coverage for aerobic and anaerobic pathogens.

Infections of the lacrimal apparatus

The lacrimal glands, accessory glands, puncta, canaliculi, tear sac, and nasolacrimal duct together are known as the *lacrimal apparatus*. Disorders and infections of the lacrimal apparatus are caused by blockage or underproduction or overproduction of tears. Inflammation of the lacrimal or tear sac—**dacryocystitis**—is the most common infection of the lacrimal apparatus. Infections are usually seen in infants and are associated with obstruction of the nasolacrimal sac. Infections are characterized by tearing and discharge from the eye, an inflamed sac, and pain (Fig. 41.11). Any organism that colonizes the nasolacrimal sac could be responsible for lacrimal sac infections (Box 41.6). *C. trachomatis* may cause a recurrent, chronic inflammation of the tear sac. *Aspergillus* spp., *Candida* spp., and *Actinomyces* spp. may also be recovered.

Dacryoadenitis, inflammation of the main lacrimal gland, may be infectious or noninfectious. Organisms are seeded into the gland via the bloodstream. Blunt trauma also predisposes the gland to infection. Common bacterial isolates include *N. gonorrhoeae*, *S. aureus*, and *Streptococcus* spp. Chronic bacterial infections of the gland involve tuberculosis, syphilis, or leprosy (Hansen disease). Mucormycosis and aspergillosis can result from contiguous spread from infections of the orbit. Mumps and infectious mononucleosis are the common viral



Fig. 41.11 Dacryocystitis (infection of the lacrimal sac) of the left eye (arrow) in a young patient. (From Zitelli, B. J., & Davis, H. W. (2008). *Atlas of pediatric physical diagnosis* (5th ed.). Philadelphia: Mosby.)

infections associated with the lacrimal gland. Subclinical or inapparent infections have occurred in patients with HSV, VZV, CMV, coxsackievirus A, and echoviruses. Other viruses implicated in lacrimal gland infections include measles and influenza viruses.

Canaliculitis, a disease found exclusively in adults, is a low-grade inflammation that affects the lower canaliculus more than the upper canaliculus. Purulent, cheesy material may be expressed from the lumen. The causative agents are diverse and include bacteria, fungi, and viruses. Bacterial infections consist of mixed aerobic and anaerobic species; *S. aureus* and streptococci are among the aerobes recovered (see Box 41.6). Aerobic isolates include gram-negative bacilli and *C. trachomatis*. The predominant anaerobic pathogens recovered include *Actinomyces israelii*, *Propionibacterium propionicus*, *C. acnes*, *Nocardia*, and *Fusobacterium*. Fungal isolates include *C. albicans* and *Aspergillus* spp. HSV and VZV may also inflame the canaliculi.

Materials for microbiological evaluation include drainage material, pus, and exudate. Direct smears and cultures for bacteria and fungi should be prepared from these materials. The causative agents (e.g., *Actinomyces* spp.) including fungal hyphae are often seen in large numbers. Infections with mixed organisms are also evident. Media should be inoculated to recover aerobic and anaerobic bacteria and fungi.

BOX 41.6 Microorganisms associated with lacrimal apparatus infections

Bacteria

Actinomyces israelii
Capnocytophaga spp.
Chlamydia trachomatis
Fusobacterium spp.
Haemophilus influenzae
Mycobacterium tuberculosis
Mycobacterium leprae
Pseudomonas aeruginosa
Proteus mirabilis
Cutibacterium acnes
Staphylococcus aureus
Streptococcus pneumoniae
Streptococcus pyogenes

Viruses

Coxsackie virus A
 Cytomegalovirus
 Echoviruses
 Epstein-Barr virus
 Herpes simplex virus types 1 and 2
 Influenza virus
 Measles virus
 Varicella-zoster virus

Fungi

Aspergillus spp.
Candida albicans
Rhizopus spp.
Mucor spp.

Parasites

Cysticercus cellulosae
Onchocerca volvulus

Infections of the intraocular chambers

Infectious **endophthalmitis**, inflammation of intraocular tissues or cavities, is a catastrophic complication of surgery, contiguous spread of infection from infected tissues, use of contaminated medications, or penetrating ocular trauma. Endophthalmitis may also be caused by instillation of contaminated eye drops and implantation of contaminated biomaterials. It is the most serious and sight-threatening of all ocular infections. Any organism that gains entry into the inner chambers of the eye can result in disease. The causative agents include bacteria, viruses, fungi, and parasites (Box 41.7).

Bacteria

S. epidermidis and other coagulase-negative staphylococci are the most frequent pathogens. Contamination of intraocular chambers and/or the intraocular lens with conjunctival microbiota is the common reservoir for these infections. Bacterial endophthalmitis is often associated with infected biomaterials (e.g., intraocular lens, scleral buckles) following cataract and/or retinal surgery. *Bacillus cereus* and other *Bacillus* spp. are the organisms most frequently isolated from patients with endophthalmitis resulting from traumatic injury. The onset of

symptoms is sudden, and the course is fulminant. Release of necrotizing enzymes can result in loss of the eye within 48 hours. A high index of suspicion followed by aggressive therapy is required to ensure that patients retain useful vision.

Fungi

Mycotic endophthalmitis is mostly an extension of keratitis. However, it can also result from hematogenous spread from a remote focus and from implantation of contaminated intraocular lenses. The most frequent isolates are *C. albicans* and other *Candida* spp. Saprophytes that infect the cornea may extend into the intraocular cavities. The filamentous species recovered include *Aspergillus* spp., *Fusarium solani*, *Paecilomyces* spp., *Curvularia* spp., and *S. schenckii*. Other reported species recovered in cases of endophthalmitis include *Monosporium apiospermum*, *Cephalosporium* spp., *Volutella* spp., *C. immitis*, *H. capsulatum*, *C. neoformans*, and *B. dermatitidis*. *Pneumocystis jirovecii* may be isolated from patients with human immunodeficiency virus (HIV) infection. Blood cultures growing fungus warrant an ophthalmologic examination to evaluate for evidence of fungal endophthalmitis and/or retinitis.

Parasites

Intraocular parasites usually affect the retina or choroid or are transient invaders from adjacent ocular structures. *Onchocerca* spp., *Toxocara* spp., and *Toxoplasma gondii* are the most frequent intraocular pathogens.

BOX 41.7 Microorganisms associated with endophthalmitis

Bacteria

Achromobacter xylosoxidans
Actinomyces spp.
Bacillus spp.
Capnocytophaga spp.
 Enterobacterales
Enterococcus faecalis
Haemophilus influenzae
Moraxella spp.
Mycobacterium chelonae
Cutibacterium acnes
Proteus mirabilis
Pseudomonas aeruginosa
Serratia marcescens
Staphylococcus aureus
Staphylococcus epidermidis and other coagulase-negative staphylococci
 Viridans group streptococci

Viruses

Cytomegalovirus
 Enteroviruses
 Herpes simplex virus types 1 and 2
 Varicella-zoster virus

Fungi

Aspergillus spp.
Candida spp. (*Candida albicans* most common)
Cryptococcus neoformans
Curvularia spp.
Fusarium spp.
Paecilomyces spp.

Parasites

Onchocerca volvulus
Toxoplasma gondii
Toxocara spp.

Laboratory diagnosis

Rapid recovery and identification of the invading organism complemented by early, specific, and aggressive therapy are mandatory to prevent loss of useful vision and preserve internal ocular structures. Specimens include aspirated anterior chamber or vitreous fluids (usually <5 mL) and washings from the flushing out of vitreous chambers (usually >10 mL). Because the volumes are generally small, intraocular fluids are plated directly onto selected media. Chocolate agar should be used, and other media are selected for other pathogens after consultation with the ophthalmologist. Drops are placed on slides for Gram, Giemsa, or calcofluor white stain. Vitreous washings are filtered through a 0.45- μ m Teflon filter, the filter is sectioned, and sections are placed on selected media (Fig. 41.12).

Retinitis

Inflammation of the retina is often associated with viral infection, such as HSV, VZV, and CMV. Fungi such as *Candida* spp. also can cause infection of the retina. No ophthalmic samples are acquired. This is diagnosed by ophthalmologic examination with possible blood culture.

Biofilm-centered ocular infections

Microorganisms in biofilms (see Chapter 31) are causative agents in a variety of ocular infections (Table 41.2). These include contact lens-associated and crystalline keratitis, late-onset postoperative endophthalmitis, and orbital implant infections. Biomaterial-centered infections have also been

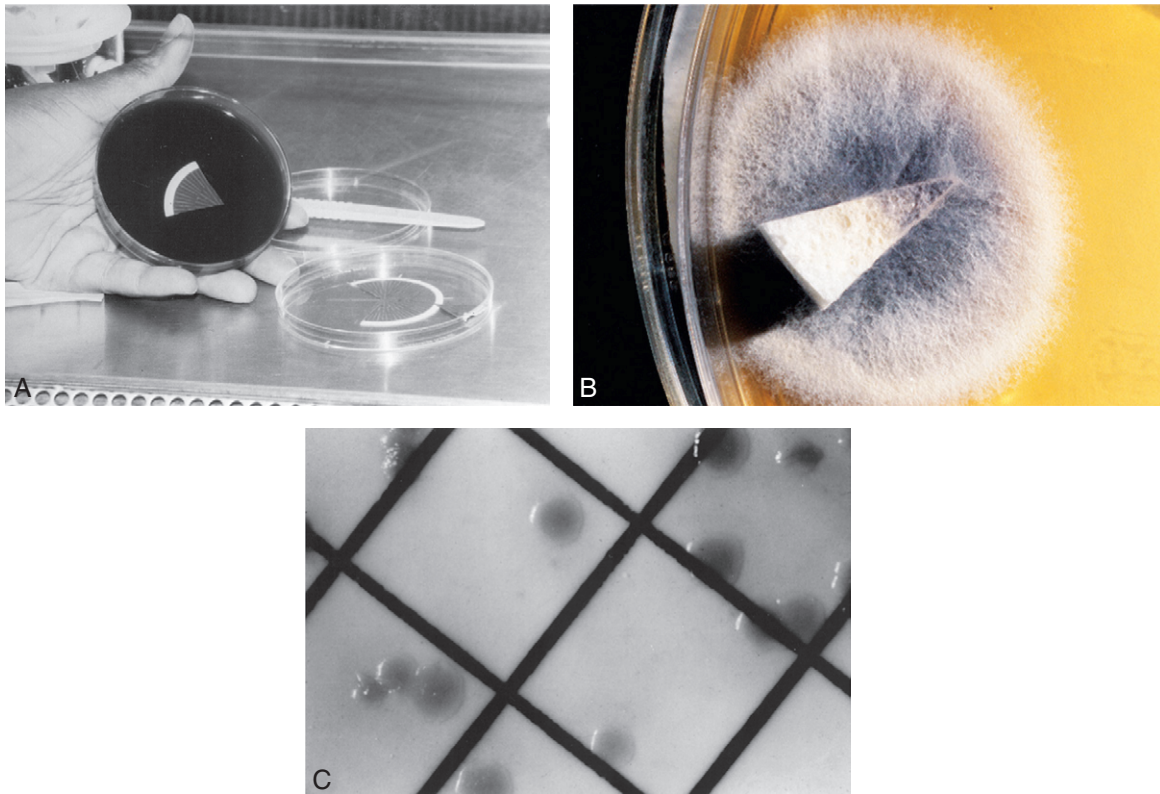


Fig. 41.12 **A**, Section from a filter used to concentrate vitreous fluids. Once the fluids have been filtered, the 0.45- μ m filter is sectioned, and sections are placed on selected media. **B**, *Curvularia* spp. from intraocular fluids on a 0.45- μ m filter section. **C**, *Burkholderia cepacia* on a filter from a vitrectomy specimen.

Table 41.2 Biofilm-associated ocular disease

Clinical disease	Biomaterial and/or biofilm origin	Recovered or common organisms
Contact lens–associated keratitis	Hydrogel and silicone (soft) contact lens PMMA (hard) contact lens	<i>Pseudomonas aeruginosa</i> <i>Staphylococcus aureus</i> Coagulase-negative staphylococci
Crystalline keratitis	Damaged tissue	Viridans group streptococci <i>Candida</i> spp.
Diffuse lamellar keratitis	Damaged tissue	Endotoxins, microbial products released from biofilms
Radial keratotomy	Damaged ocular tissue	Viridans group streptococci
Intracorneal rings	Damaged tissue implant	<i>Serratia marcescens</i> <i>Pseudomonas aeruginosa</i>
Endophthalmitis	Intraocular lenses	Coagulase-negative staphylococci (<i>Staphylococcus epidermidis</i> —most frequent) <i>Cutibacterium acnes</i> <i>Candida albicans</i>
Orbital implant infections	Implant Orbital tissues	<i>S. aureus</i>
Glaucoma drainage	Implant Damaged tissue	<i>S. aureus</i> <i>Mycobacterium</i> spp.
Punctual plugs	Implant Damaged tissue	<i>S. epidermidis</i>
Lacrimal plugs	Implant Damaged tissue	<i>Mycobacterium chelonae</i> <i>S. aureus</i>

PMMA, Polymethylmethacrylate.

documented for ocular keratoprosthesis, glaucoma shunts, and ocular sutures. Biofilms can form on a variety of ocular biomaterials and/or on damaged ocular tissues. The ocular biomaterials most frequently predisposed to biofilm formation include contact lenses, intraocular lenses, and corneal and orbital implants. Common pathogens include coagulase-negative staphylococci, *S. aureus*, *C. acnes*, *C. albicans*, and *P. aeruginosa* (Fig. 41.13). Biofilm-centered ocular infections are recalcitrant to antimicrobial therapy, and the sequestered biofilm-encased organisms are protected against the host's humoral and cellular immunity. Complications include chronic infections, decreased vision, and blindness. Removal of the biomaterial is often necessary to resolve the infection or inflammation.

Laboratory diagnosis of ocular infections

Specimen collection

The keys to proper collection of ocular specimens are similar to those for any other microbiological specimen. Materials or

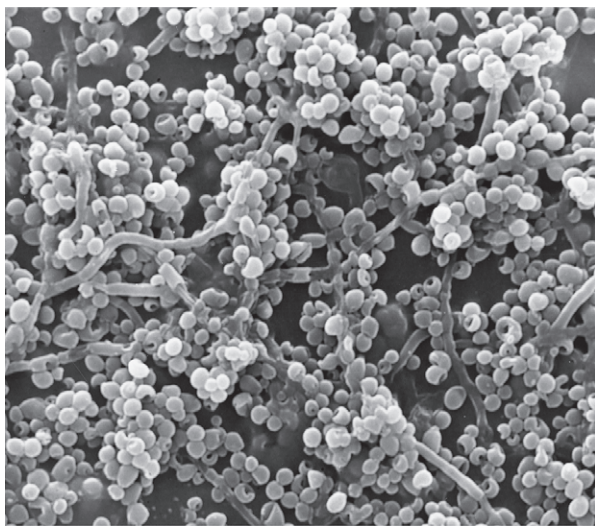


Fig. 41.13 Fungal (yeast) biofilm on a contact lens. The culture grew *Candida albicans*.

scrapings for cultures should be collected as soon as possible after the onset of infection (24 to 48 hours for bacteria and 3 to 7 days for viruses) and before the instillation of antimicrobials or steroids. The sample must be collected from the actual site of the infection; for example, conjunctival and eyelid cultures are inadequate to assess corneal involvement. All ocular fluids, tissues, sponges, and other surgical materials must be submitted in sterile, leakproof containers that are properly labeled.

The ophthalmologist must communicate with the laboratory scientists performing the microbiological evaluation of the specimen to ensure isolation of ocular pathogens. It should be noted whether the patient is a contact lens wearer. For best results, ocular materials must be inoculated directly onto appropriate media. Scant recovery is the norm when transport swabs are submitted for recovery of ocular pathogens. The type of swab used can further reduce organism recovery. Cotton-tipped swabs inhibit the growth of some bacteria and HSV because of the fatty acids released during sterilization. Dacron or calcium alginate swabs may be used to collect ocular samples. However, calcium alginate swabs may inactivate some viral pathogens.

Materials expressed from areas such as canaliculi are preferred to rule out infection (Fig. 41.14). Materials are inoculated directly onto media. Smears are also prepared from the materials. Collection by direct aspiration or scraping is performed by the ophthalmologist. Media to recover aerobic and anaerobic organisms must be included. Vitreous washings (with a volume >10 mL) may be injected into blood culture bottles or viral transport media or sent to the laboratory for concentration through a 0.22- or 0.45- μ m filter (see Fig. 41.12). Biopsy tissue must be minced or ground. Media to recover aerobic and anaerobic bacteria, fungi, and mycobacteria should be inoculated. If amebae are suspected, two nonnutrient agar plates should be included.

Direct microscopic examination

Conjunctival and corneal scrapings, intraocular fluids, and aspirates are collected by an ophthalmologist using a Kimura spatula, blade, or sterile swab. Scrapings and fluid aspirates from the involved ocular site complemented with the appropriate stains, can give the health care provider circumstantial and definitive information concerning the identity of the invading organisms. Table 41.3 provides a summary of stains

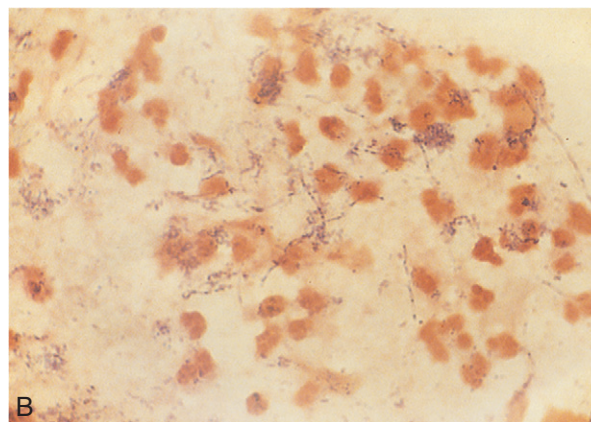
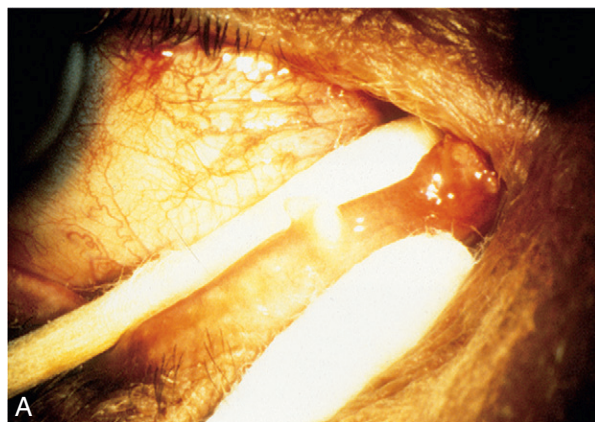


Fig. 41.14 A, Concretions being expressed from canaliculi. B, Smashed and stained concretions, revealing gram-positive, slender, branching rods (*Actinomyces israelii*).

that are routinely used in ocular microbiology and appropriate applications of each procedure. Gram and Giemsa stains (Fig. 41.15) should be used for all suspected cases of bacterial conjunctivitis, keratitis, and endophthalmitis. Calcofluor white stain should be added to rule out fungal or parasitic disease. A fluorescent or acid-fast stain is used to detect the presence of acid-fast organisms. In addition, impression

cytology using 0.45- μ m Teflon-coated membrane filters to collect conjunctival and corneal tissue may increase bacterial, fungal, viral, and protozoal detection by as much as 50%. The advantage of this technique is that cells remain intact, with characteristic morphology and microbial infestation and invasion (Fig. 41.16).

Culture and identification

Most bacterial and fungal ocular isolates may be recovered on chocolate and blood agar when incubated under the proper conditions of 35° C and 5% to 10% carbon dioxide. Additional plates should be incubated anaerobically. Table 41.4 shows a general plating guide that may be followed to recover typical agents of ocular infections. The addition of thioglycollate broth, Thayer-Martin agar (for the pathogenic *Neisseria*), Sabouraud dextrose agar with gentamicin (Fig. 41.17), Löwenstein-Jensen slants, and non-nutrient agar plates allows the recovery of most pathogens involved in ocular disease. All thioglycollate tubes are held for 10 days or for 21 days if there is suspicion of *Actinomyces* spp. or *C. acnes*. The fungi that traditional mycology media aim to inhibit (saprophytes) are the agents usually recovered in mycotic ocular disease (e.g., *Fusarium* spp., *Paecilomyces* spp., *Aspergillus* spp., *Penicillium* spp.). Therefore media with cycloheximide should be avoided. Very little clinical material is often available; media should be inoculated at the bedside. Vitreous humor should be centrifuged and the sediment used for media and smears.

Interpretation of growth from ocular samples is based on the same sound microbiological criteria used in general hospital microbiology laboratories. Quantitation of growth is particularly important. Samples from patients receiving antimicrobial agents, steroids, or other medications may have reduced microbial growth, which should be considered when evaluating the culture. Special considerations are involved in corneal and intraocular fluid cultures. Traditionally, corneal scrapings are inoculated in a C-streak fashion (Fig. 41.18). Each row of C streaks represents a separate scraping of a corneal ulcer. The dilution effect is from left to right. Generally, more colonies of bacteria or fungi appear on the first C streaks of each row. Each successive C streak progresses from the

Organism	Gram	Giemsa	IF	IC	CFW
Bacteria	+	+			
Fungi ^a	+	+			+
Viruses		+	+	+	
Chlamydia		+	+	+	
Parasites	+	+		+	+

^aThe tissue Gram stains Brown-Brenn and Brown-Hopps or the Warthin-Starry silver stain are preferred for microsporidia.

CFW, Calcofluor white stain (used to detect fungi or parasites in ocular tissue); IC, impression cytology (collected cells remain intact, and test can track disease progress); IF, immunofluorescent stains (monoclonal antibodies for chlamydia, herpes simplex virus, adenovirus, enterovirus, varicella-zoster virus, and cytomegalovirus).

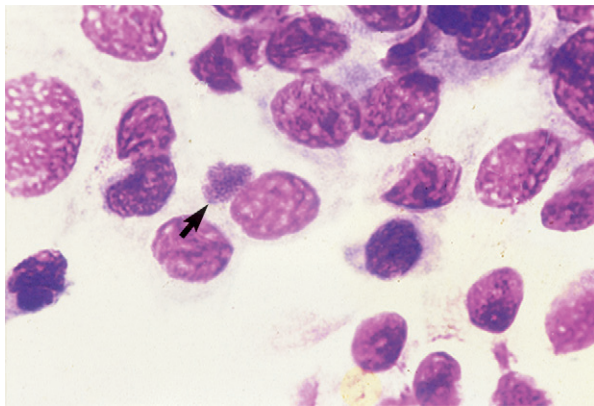


Fig. 41.15 Giemsa-stained smear of conjunctival epithelial cells with chlamydial inclusions (arrow). The Giemsa stain also provides information on the types and numbers of inflammatory cells and the condition of the epithelial cells.

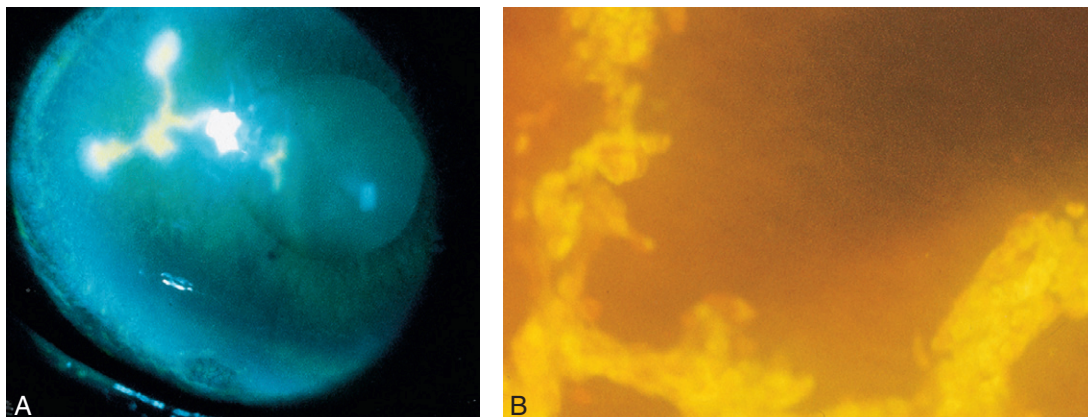


Fig. 41.16 **A**, Cornea stained with rose bengal stain to outline a dendrite infected with herpes simplex virus (HSV). HSV is the virus most often isolated from corneal dendritic infections. **B**, Dendrite on a membrane filter collected by impression cytology. The filter was stained with a monoclonal antibody against HSV-1. Almost all cells of the dendrite fluoresced when stained. The uninfected cells do not stain and appear red.

Table 41.4 General ocular plating guide^a

Clinical presentation	Minimum media to inoculate									Stains				Common isolates
	Chocolate agar	5% Sheep blood agar	Sabouraud agar	Thioglycollate	Agar/agar	Anaerobic blood agar	Löwenstein-Jensen slant	Blood culture bottles	Viral and/or <i>Chlamydia</i> transport media	Gram stain	Giemsa stain	Calcofluor white stain	Immunofluorescent stain	
Conjunctivitis														
Bacterial	X	X								X				<i>Staphylococcus aureus</i> , <i>Haemophilus influenzae</i> , <i>Streptococcus pneumoniae</i>
Chlamydial	X								X	X	X		X	<i>Chlamydia trachomatis</i>
Viral	X								X				X	Adenovirus, HSV-1, HSV-2, Enterovirus
Blepharitis														
Same as for conjunctivitis														Same as for conjunctivitis
Keratitis														
Bacterial	X	X		X						X				<i>Pseudomonas aeruginosa</i> , <i>S. aureus</i> , <i>Serratia marcescens</i>
Fungal	X	X	X	X						X	X	X		<i>Fusarium</i> spp., <i>Candida</i> spp., <i>Aspergillus</i> spp.
Viral	X								X				X	HSV-1, HSV-2, VZV, adenovirus
Free-living amoeba	X	X			X					X		X		<i>Acanthamoeba</i> spp., <i>Vahlkampfia</i> spp.
Contact lenses	X		X		X							X		<i>P. aeruginosa</i> , <i>S. aureus</i> , <i>amebae</i>
Atypical	X	X	X	X	X	X	X			X	X			MOTT, <i>Nocardia</i> spp.
Endophthalmitis														
Bacterial	X	X		X		X		X		X				CoNS, <i>S. aureus</i> , <i>Cutibacterium acnes</i>
Fungal	X	X	X	X						X				<i>Candida</i> spp., <i>Aspergillus</i> spp.
Viral	X								X				X	HSV, VZV, CMV (NAAT preferred)
Orbital cellulitis														
Bacterial	X	X		X		X				X				<i>S. aureus</i> , anaerobes, <i>H. influenzae</i>
Fungal	X		X	X						X				<i>Aspergillus</i> spp., <i>Mucor</i> spp., <i>Rhizopus</i> spp.
Preseptal cellulitis														
Same as for orbital cellulitis														
Dacryocystitis														
Bacterial	X	X		X		X				X				<i>S. aureus</i> , <i>Actinomyces</i> spp., anaerobes
*Additional procedures include acid-fast bacillus stains, DNA probes, <i>Limulus</i> lysate, and filtration. CMV, Cytomegalovirus; CoNS, coagulase-negative staphylococci; HSV, herpes simplex virus; MOTT, mycobacteria other than tubercle bacilli; NAAT, nucleic acid amplification test; VZV, varicella-zoster virus.														

^aAdditional procedures include acid-fast bacillus stains, DNA probes, *Limulus* lysate, and filtration.

CMV, Cytomegalovirus; CoNS, coagulase-negative staphylococci; HSV, herpes simplex virus; MOTT, mycobacteria other than tubercle bacilli; NAAT, nucleic acid amplification test; VZV, varicella-zoster virus.



Fig. 41.17 Sabouraud agar plate with mold and yeast. The patient had a mixed fungal keratitis. *Top*, The superficial layer of the cornea was infected with a mold (*Fusarium oxysporum*). *Bottom*, The deeper layers were infected with a yeast (*Candida albicans*).

superficial to deep layers of the cornea. The greater the number of corneal streaks with growth, the more involved and serious is the infection.

Any growth from intraocular fluids appearing on the inoculation sites or filter should be assessed and reported. The health care provider correlates the organism's pathology with the patient's clinical picture and diagnosis. Coagulase-negative staphylococci, which are the most common isolates and which are normally considered contaminants, are the ones most frequently involved in microbial intraocular infections (Fig. 41.19). Staining, impression cytology, and tissue culture are routinely used to confirm ocular chlamydial and viral pathogens.

Molecular techniques

Direct antigen detection and NAATs often provide the most rapid and accurate identification, especially in confirming clinical diagnosis for difficult or unusual ocular pathogens. These methods may be ideal for detecting microbes in ocular samples because of the small volume. Molecular techniques have been used to detect *Chlamydia*, herpesviruses, and *Toxoplasma*.

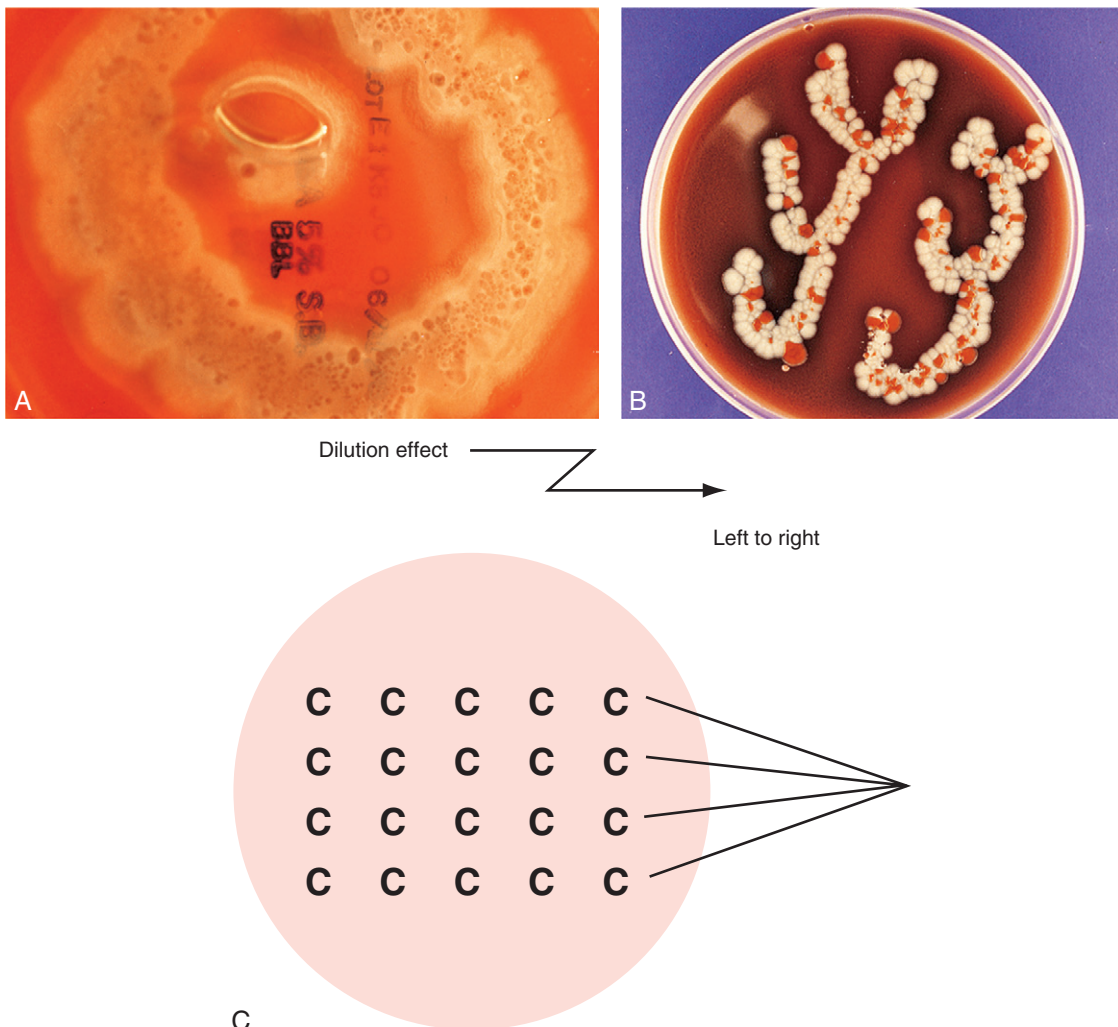


Fig. 41.18 **A**, Contact lens and lens solution on 5% sheep blood agar surrounded by growth of *Pseudomonas aeruginosa*. **B**, C streaks growing pigmented and nonpigmented *Serratia marcescens*. **C**, Corneal scrapings. Note that each row of C streaks represents a separate corneal scraping.

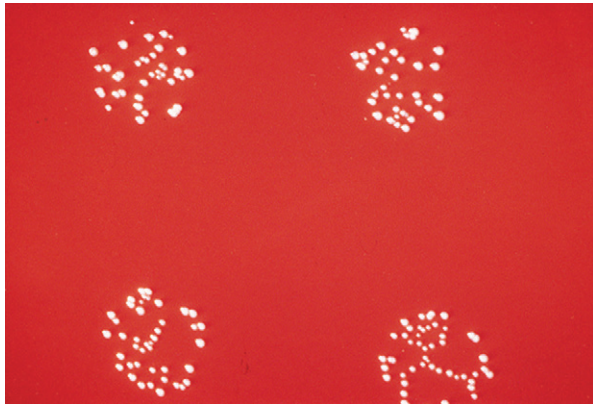


Fig. 41.19 *Staphylococcus epidermidis* recovered from vitreous fluids (drops) on a blood agar plate. Samples may be inoculated onto a chocolate or blood agar plate and allowed to dry or may be streaked as for a routine microbiology specimen.

BOX 41.8 Microbes recovered from contaminated ocular medications

Acanthamoeba spp.
Alcaligenes spp.
Aspergillus spp.
Candida spp.
Citrobacter freundii
 Coagulase-negative staphylococci
Enterobacter cloacae
Klebsiella spp.
Micrococcus spp.
Morganella morganii
Mycobacterium chelonae
 Nonglucose-fermenting gram-negative rods
Ochrobactrum anthropi
Proteus mirabilis
Pseudomonas aeruginosa and other *Pseudomonas* spp.
Serratia spp.
Staphylococcus aureus
Stenotrophomonas maltophilia
Mucor
Rhizopus

BOX 41.9 Microorganisms recovered from contact lenses, contact lens solutions, and contact lens cases

Acanthamoeba spp.
Achromobacter spp.
Acinetobacter baumannii complex
Aeromonas spp.
Agrobacterium tumefaciens
Alcaligenes spp.
Bacillus spp.
Brevundimonas vesicularis
Burkholderia cepacia
Candida spp.
Chryseobacterium spp.
Citrobacter spp.
Comamonas spp.
Enterobacter spp.
Escherichia coli, *Escherichia hermannii*
Flavimonas spp.
Flavobacterium spp.
Fusarium spp.
Gemella spp.
Haemophilus spp.
Klebsiella spp.
Mycobacterium spp.
Neisseria spp.
Ochrobactrum anthropi
Paecilomyces spp.
Propionibacterium spp.
Proteus spp.
Pseudomonas aeruginosa and other *Pseudomonas* spp.
Serratia spp.
Staphylococcus aureus
Staphylococcus epidermidis and other coagulase-negative staphylococci
Stenotrophomonas maltophilia
Streptococcus pneumoniae
Trichoderma spp.
Vahlkampfi and other free-living amoebae
 Viridans group streptococci

Special culture techniques

Contaminated ocular medications

Ophthalmic drops (e.g., antimicrobials, analgesics) are easily contaminated when improperly handled by patients or ophthalmic clinic personnel. Inappropriate handling can contribute to ongoing ocular disorders and initiation of new infections. Even though most ocular medications are prepared with preservatives to inhibit the growth of microorganisms, once the preservative's threshold has been breached, microbes survive, multiply, and are dispensed with the next drop of medication. Medications should be collected from patients with conjunctivitis, keratitis, or endophthalmitis and sent to the laboratory for culture. The medications that are usually contaminated and associated with concurrent patient infection include steroids, beta blockers, antiglaucoma drugs, antimicrobials, and artificial tears. [Box 41.8](#) lists the microbes recovered from contaminated ocular medications.

Contact lenses and solutions

As mentioned previously, contact lenses are major risk factors for microbial keratitis. Keratitis associated with contact lenses (soft, daily wear, extended wear, or disposable) is being reported with increasing frequency. Sources include poor hygiene with handling and maintenance of contact lenses and contact lens solutions and cases. [Box 41.9](#) lists microorganisms recovered from these sites. Contamination of soft contact lenses is usually caused by failure to follow the manufacturer's recommended procedures or by poor hygiene. Soft lenses contain a large percentage of water, allowing pathogens to survive and multiply. Organisms form and attach to biofilms on the contact lens case, contact lenses, or tissue. Placement of the lenses on the cornea delivers a high microbial load and facilitates attachment and entrance into the cornea. The cornea stroma serves as an excellent medium for microbial proliferation and spread. Wearers of hard contact lenses are less susceptible to infection from microbial contamination, which might be related to the type of biomaterials used to make this type of lens.

Cornea storage media and tissue culture

Replacement of diseased or opacified corneas is a common operation performed by ophthalmic surgeons. The new corneal tissue is obtained from donors within 24 hours of death and is preserved in McCarey and Kaufman medium or Dexsol medium. After surgery, the container with the medium and remaining corneal rim is sent to the laboratory for culture. Bacteriologic evaluation of the donor tissue is paramount in reducing the transmission of host-carried disease to the recipient. Viral studies of corneal transplantation tissue have been limited.

Gram-positive organisms, including coagulase-negative staphylococci and *C. acnes*, are the most frequently isolated organisms, but gram-negative organisms and fungi may also be recovered from these media. The actual correlation between isolates and resultant disease is less than 1%. All positive cultures should be reported directly to the surgeon and a final report sent to the eye bank and the health care provider.

POINTS TO REMEMBER

- The external ocular surface is constantly challenged by microbes.
- Ocular infections may be mild or sight-threatening.
- Surface defenses and an intact cornea and globe prevent invasion by most microorganisms.
- Any organism gaining entry to the eye can establish infection.
- Inflammatory response can damage ocular structures.
- Contact lens wear is considered the greatest risk factor for developing microbial keratitis.
- *P. aeruginosa*, *S. aureus*, coagulase-negative staphylococci, *H. influenzae*, and *S. pneumoniae* are bacteria commonly associated with eye infections.
- Infections of the cornea and intraocular structures are medical emergencies.

LEARNING ASSESSMENT QUESTIONS

1. What is the most common gram-positive ocular pathogen?
 - a. *Cutibacterium acnes*
 - b. *Staphylococcus aureus*
 - c. *Corynebacterium* spp.
 - d. Coagulase-negative staphylococci
2. Media for the isolation of fungal pathogens from ocular specimens should not contain which component?
 - a. Gentamicin
 - b. Penicillin
 - c. Cycloheximide
 - d. Streptomycin
3. Which uncommon pathogen found in water, is associated with contact lens use, and can invade corneal tissue?
 - a. *Haemophilus influenzae*
 - b. *Penicillium* spp.
 - c. *Fusarium* spp.
 - d. *Acanthamoeba*
4. Which group of viruses is most frequently involved in ocular disease?
 - a. Herpesviruses
 - b. Adenoviruses
 - c. Enteroviruses
 - d. Poxviruses
5. Nonnutrient agar inoculated with *Escherichia coli* is used to recover which organism from ocular specimens?
 - a. Microsporidia
 - b. *Chlamydia*
 - c. *Fusarium* spp.
 - d. *Acanthamoeba*
6. Requests for culture from which ocular site are received most often? Which organisms are most likely to be recovered?
7. Which stains are used commonly on smears to identify ocular pathogens?
8. What is the most common route of administration for the antimicrobial treatment of uncomplicated conjunctivitis?
9. Which three infectious agents cause sexually transmitted diseases that can also affect the eye?
10. Which procedure is of the most value when performing bacterial cultures to diagnose conjunctivitis?
11. Why are contact lenses a risk for eye infection, and how can this be reduced?
12. Bacteria that cause keratitis associated with contact lenses often initiate infection through which of the following?
 - a. Biofilm formation
 - b. Microbial growth in the contact lens
 - c. Attachment of bacteria to the lens
 - d. Contamination of the lens fluid
13. A patient visits her health care provider because of a blood-red right eye that appears infected. She recently bought a new pair of contact lenses to change the color of her eyes. The health care provider orders bacterial, fungal, and viral cultures, all of which are negative. What is the most probable cause of this infection?
 - a. *Naegleria* spp.
 - b. *Acanthamoeba* spp.
 - c. *Entamoeba* spp.
 - d. *Trichinella* spp.
14. Which condition is considered a true ocular emergency?
 - a. Conjunctivitis
 - b. Blepharitis
 - c. Microbial keratitis
 - d. Scleritis
15. Which parasite is responsible for causing a form of blindness in West Africa?
 - a. *Loa loa*
 - b. *Brugia malayi*
 - c. *Onchocerca volvulus*
 - d. *Wuchereria bancrofti*

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Answers to learning assessment questions

Chapter 1

1. The Gram stain must be repeated because the *Escherichia coli* quality control slide gave a false gram-positive reaction. Because of this quality control result, the smear made from the clinical sample could not be accurately interpreted.
2. The most likely cause of the false gram-positive result for *E. coli* was insufficient decolorization time. Crystal violet, the primary stain, was not removed from the bacterial cells.
3. Pili, made up of pilin protein, aid in bacterial attachment to solid surfaces such as mucous membranes. Flagella, consisting of flagellin protein, are responsible for motility.
4. Capsules help microorganisms resist phagocytosis. Phagocytic cells are less able to bind to the capsular polysaccharide than they are to surface proteins. In addition, capsules hide antigens on the surface of bacteria, preventing them from interacting with antibodies produced by an animal in response to an infection.
5. LPS is also known as *endotoxin*. This molecule is toxic to animals, inducing nonspecific effects such as fever, inflammation, hypotension, and shock. LPS contains three regions: an antigenic O-specific polysaccharide, a core polysaccharide, and an inner lipid A. The lipid A moiety is responsible for producing fever and shock conditions in patients infected with gram-negative bacteria.
6. c
7. a
8. b
9. Older bacterial cells are decolorized more easily than younger cells because as cells age, their cell walls become “leaky” and allow molecules to pass more readily out of the cell. In the Gram stain, the crystal violet-iodine complex is more readily lost during the decolorization step.
10. Bacterial spores have a thick protein coat that makes them highly resistant to chemical agents, temperature changes, dehydration, ultraviolet and gamma radiation, and desiccation. They are also metabolically inactive and can therefore survive periods of starvation for years.
11. The three ways in which genetic material may be transferred from one bacterium to another are transformation, transduction, and conjugation. Transformation is the uptake and incorporation of naked or free DNA into a bacterial cell. Transduction is the transfer of bacterial genes by a bacteriophage from one cell to another. Conjugation is the transfer of genetic material from a donor bacterial strain to a recipient strain. Conjugation requires close contact between the two cells.
12. 3' TTACGGACAAC 5'
5' AATGCCTGTTG 3'
13. Uracil
14. During lysogeny, phage DNA is inserted into the bacterial genome, but virus particles are not produced. When the bacterial cell replicates, the viral genome is copied along with the bacterial chromosome. During the lytic cycle, bacteriophages are produced until the bacterial cell dies and is lysed.
15. a
16. c
17. a
18. b

Chapter 2

1. c
2. a
3. b
4. d
5. b
6. a
7. d
8. c

9. a
10. b
11. Resident microbiota are organisms that occupy a specific body site for a long time (months to years). Transient biota are those microorganisms that inhabit a site for a short time, or temporarily.
12. The carrier is a host, often asymptomatic, of an infection who can transmit the infecting organism or virus to a susceptible individual.
13. The nutritional status of the site, pH, oxidation-reduction potential, and presence and interference of already established organisms all determine the composition of indigenous biota.
14. The resident microbiota helps: (1) prime the host immune system; (2) produce substances toxic to more pathogenic species; and (3) decrease the numbers of pathogenic organisms by competing for nutrients.
15. Virulence factors produced by *Clostridioides difficile* include toxins A and B. Toxin A acts as an enterotoxin; toxin B acts as a cytotoxin.
16. True pathogens are organisms that cause disease in susceptible hosts a high percentage of the time. An opportunistic pathogen is an organism that can be a member of the indigenous biota of the host. When the host immune system is compromised or changed, the organism takes the opportunity to cause disease.
17. Exotoxins are extracellular substances produced by an organism containing a toxin gene often encoded by phages, plasmids, or transposons. Toxins generally have a specific activity. Endotoxins compose the lipopolysaccharide component of the cell wall of gram-negative bacteria. The toxicity is caused by the lipid A portion of lipopolysaccharide.
18. The following table compares the cells and soluble mediators involved in the innate immune response, the adaptive humoral immune response, and the cellular immune response.

	Cells	Soluble Mediators
Innate immune response	Macrophages Natural killer cells Neutrophils	Complement Cytokines
Humoral immune response	T-helper cells B cells	Antibodies
Cellular immune response	Cytotoxic T cells Helper T cells Macrophages Natural killer cells	Lymphokines

19. The major immune response against extracellular bacteria is antibodies binding and inducing complement-mediated lysis and phagocytosis. The major immune response against intracellular bacteria is cell-mediated immunity, in which TH1 cells activate macrophages to phagocytose and destroy infected cells. The major immune response against viruses is cytotoxic T cells and natural killer cells that target virus-infected cells. Antibodies are only effective in preventing viruses from attaching to and entering host cells.

20. The following are the steps involved in phagocytosis and how microorganisms can evade each step:
 - Contact between the phagocytic cell and the microorganism. Organisms can evade this step by preventing opsonization with antibody and complement and by masking its antigens with a capsule.
 - Formation of the phagosome. The phagocytic cell extends pseudopodia around the microorganism. The microorganism is contained with a membrane (phagosome).
 - Fusion of the phagosome with lysosomes to form a phagolysosome. Organisms can evade this step by leaving the phagosome and residing in the cytoplasm or preventing fusion of the phagosome and lysosomes.
 - Digestion of the organism. Some organisms secrete enzymes such as catalase and superoxide dismutase that break down the oxygen-reactive intermediates that are present to kill and digest the organism. The production of these enzymes makes the organism resistant to digestion. Only a few bacteria can survive inside phagolysosomes.

Chapter 3

1. a
2. b
3. b
4. c
5. d
6. c
7. d
8. b
9. d
10. d

Chapter 4

1. Sterilization is a chemical or physical process resulting in the destruction of all living organisms. Disinfection can be either a physical or chemical method that reduces the number of viable cells.
2. Disinfectants are frequently used in hospitals, dental surgery centers, kitchens, and bathrooms to kill infectious organisms on inanimate objects. They are used after cleaning for surfaces that have visible blood or drainage from infected skin. They are also used to clean surfaces such as toilets, sinks, floors, drains, doorknobs, and countertops. Disinfectants aid in maintaining a clean environment to help prevent the spread of bacteria that may cause infections. Antiseptics are used in handwashing such as before a surgical procedure or contact with those who are at high risk of infection, such as newborns. Antiseptics destroy and inhibit the growth of microorganisms on living tissues (e.g., the skin or mucous membranes). They are not as strong as disinfectants, so they should not be used to clean objects and surfaces.

3. Physical methods involve heat (e.g., boiling water, autoclaving, pasteurization, dry heat), radiation (e.g., ionizing and nonionizing), and filtration (e.g., liquid and air). Chemical methods involve both disinfectants and antiseptics. They include chemicals such as alcohols, aldehydes (e.g., formaldehyde and glutaraldehyde), halogens (e.g., iodophors), chlorine, quaternary ammonium compounds, phenolics (e.g., chlorhexidine gluconate, chloroxylenol), heavy metals, and gases.
4. High-level disinfectants (e.g., aldehydes) and autoclaving kill bacterial endospores and fungal spores.
5.
 - a. Type of organisms: Organisms vary greatly in their ability to withstand chemical and physical treatments because of their biochemical composition and various mechanisms that they can use to protect themselves.
 - b. Number of organisms: The microbial load determines the exposure time necessary for elimination of the microorganisms.
 - c. Concentration of the disinfecting agent: The amount of disinfectant needed to destroy microorganisms varies with the different agents.
 - d. Presence of organic material: Organic material such as blood, mucus, and pus affect killing activity by inactivating the disinfecting agent.
 - e. Contact time: It is critical to know what organisms may be present and the contact time to use based on the microorganism that is most resistant.
 - f. Temperature: Disinfectants as well as sterilants can be rendered inactive by too high or too low a temperature.
 - g. pH: pH can influence the activity of the disinfecting or sterilizing agent.
 - h. Biofilms: The concentration of the disinfectant may need to be increased, the contact time may need to be increased, or both.
6. The Antimicrobial Division of the EPA regulates the registration on the use, sale, and distribution of antimicrobial pesticide products for certain inanimate, hard, nonporous surfaces, or pesticide products incorporated into substances under the pesticide law, the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA). An EPA registration number is granted only when the requirement of laboratory test data, toxicity data, product formula, and label copy are approved. The disinfectant label should indicate several highlighted points important in selecting the appropriate agents for the designated use. The U.S. FDA approves antiseptics through two processes: the new drug application (NDA) process or the over-the-counter (OTC) drug review known as the *monograph system*. NDAs are defined by law as being recognized as safe and effective (RASE). The approved NDA is manufacturer-specific and allows only that particular manufacturer to market the product.
7. c
8. d
9. Alcohols inactivate microorganisms by denaturing proteins. Aldehydes denature proteins and inactive nucleic acids. Iodine and chloride compounds are active based on oxidative effects. Heavy metals (e.g., silver, mercury, zinc, copper) are less commonly used today because of their toxicity; they work by combining with proteins, thereby inactivating them. The antimicrobial action of cationic detergents (e.g., benzalkonium chloride, cetylpyridinium chloride) is mediated through disruption of the cellular membrane, resulting in leakage of cell contents. Phenolics, molecules derived from phenol, work by denaturing proteins and disrupting plasma membranes. The killing mechanism of ethylene oxide is the alkylation and subsequent inactivation of nucleic acids.
10. The main goal of handwashing is to eliminate transient microbiota. Health care workers can acquire microbes, including methicillin-resistant *Staphylococcus aureus* and SARS-CoV-2 virus, during direct contact with patients or contaminated surfaces. The purpose of the surgical hand scrub and waterless surgical hand rubs is to eliminate the transient biota and most of the resident biota. Resident biota can be persistently isolated from the hands of most people. These organisms include coagulase-negative staphylococci, *Corynebacterium* (diphtheroids or coryneforms), *Propionibacterium*, and *Cutibacterium* spp. The goal of preoperative skin preparation formulations is to disinfect an intended surgical site rapidly as well as provide a high level of bacterial inactivation and persistent antimicrobial activity, up to 6 hours after skin preparation. Preoperative skin preparation products are defined as fast-acting, broad-spectrum, and persistent antiseptic-containing preparations that significantly reduce the number of microorganisms on intact skin.
11. d
12. True
13. c
14. b
15. b
16. d
17. c
18. a
19. The NFPA hazard-rating diamond states risk for flammability (red quadrant), reactivity (yellow quadrant), and health (blue quadrant). Each criterion is rated on a scale of 0 to 4, from stable or safe to highly dangerous. The bottom white quadrant is used to indicate special hazards.
20. b
21. c
22. a
23. c
24. c
25. e

Chapter 5

1. b
2. b
3. d
4. a
5. c
6. b
7. d
8. a
9. d

10. a
11. b
12. 89.3%
13. 95%
14. Efficiency of tests
15. b

Chapter 6

1. c
2. Subcutaneous infections are below the external surface of the skin, into the connective tissues. A swab specimen will collect material only from the external cutaneous surface and will not be representative of the infectious process. The sample should be collected via needle aspiration or punch biopsy.
3. c
4. b
5. b
6. d
7. b
8. a
9. d
10. c
11. a
12. True
13. The following questions are asked as each specimen culture is examined:
 - What is the specimen source?
 - Does this source have normal biota, or is it a sterile source?
 - If normal biota is present, what bacteria are found, and what do these colonies look like?
 - What are the most likely pathogens in this specimen?
 - What is the colony morphology of these pathogens?
 - Which medium is demonstrating growth, and what is the purpose of the medium?
14. Many laboratories use the Maki roll technique to culture a vascular catheter tip. A segment of the catheter tip is rolled across the surface of a blood agar plate. The plate is incubated, and testing is performed on each organism that produces 15 or more colonies.
15. The role of the microbiology laboratory in the postanalytic process is to communicate accurate and timely information. Preliminary results are often reported as the information becomes available. The laboratory report must be clear and understandable; interpretive statements may be necessary. Critical values must be reported to the health care provider immediately.

Chapter 7

1. True
2. a
3. d
4. c
5. a
6. b
7. d

8. c
9. b
10. d
11. c

Chapter 8

1. b
2. SBA is a nonselective medium that supports the growth of gram-positive and gram-negative bacteria. MAC is a selective medium that inhibits the growth of gram-positive bacteria and allows gram-negative bacilli to grow. One of the three isolates on the SBA plate is gram-positive and cannot grow on MAC agar.
3. a
4. They generally produce clear, colorless colonies on MAC agar.
5. β -Hemolysis is complete hemolysis of the red blood cells in the agar, showing a clear zone around the colony. α -Hemolysis is incomplete hemolysis of the red blood cells, showing a green discoloration around the colony.
6. "Puffballs" in broth media usually suggest the presence of certain streptococcal species.
7. c
8. d
9. b
10. c

Chapter 9

1. a
2. d
3. b
4. c
5. d
6. a
7. b
8. d
9. c
10. a
11. Two important enzymes necessary for the rapid metabolism of lactose are lactose (β -galactoside) permease and β -galactosidase. The permease enzyme facilitates the uptake of lactose. Delayed lactose fermenters lack permease but contain β -galactosidase. It takes delayed lactose fermenters longer to transport lactose across the cell wall and plasma membrane. Acid formation can be delayed until 48 to 72 hours.
12. No color change after the addition of the reagents indicates that nitrite (NO_2) is not present. Two explanations are that the bacterium was unable to reduce nitrate (NO_3) or the bacterium reduced nitrate to nitrite and then to nitrogen gas (N_2). Zinc dust reduces nitrate to nitrite. If, after the addition of zinc dust, the broth still does not change color, then the nitrate had been totally reduced by the bacterium to nitrogen gas. If color forms after the addition of zinc dust, then the nitrate had not been reduced by the bacterium, and this is a true-negative result.

Chapter 10

1. a
2. c
3. b
4. b
5. c
6. d
7. c
8. a
9. d
10. b
11. Double immunodiffusion is a precipitation reaction performed in an agarose matrix. The antigen and specific antibody are placed in separate nearby wells, and both diffuse through the agarose. At the zone of equivalence, a visible precipitate is formed. With single radial immunodiffusion, antibody is evenly distributed in an agarose matrix. Corresponding antigen is added to wells in the agar. The antigen diffuses through the agar, and a concentration gradient is produced. At the zone of equivalence, a visible precipitate is formed. The diameter of the precipitation ring is proportional to the concentration of the antigen.
12. False-negative test results can be the result of: (1) immunocompromised patients not being able to produce detectable antibody levels; (2) the sample being collected too early in the course of the infection (in some diseases, it may take a long time to form antibodies); (3) the diagnostic kit having too high a concentration of antigen (postzone phenomenon); or (4) the presence of a heterophile antibody in the patient sample binding to the antigen in the test kit blocking specific antibody from binding.
13. During passive agglutination, an antigen is bound to a carrier particle, such as latex beads. When the carrier particles are mixed with a clinical sample, agglutination will occur if the corresponding antibody is present. With reverse passive agglutination, antibody is fixed to the surface of a carrier particle. When the carrier particles are mixed with a clinical sample containing the corresponding antigen, agglutination occurs.
14. There are two major serotypes of HIV: 1 and 2. The vast majority of cases in the United States are caused by HIV-1, while HIV-2 is found predominantly in Africa. However, because of international travel, clinical laboratories must screen for both serotypes. Because it takes most patients several weeks to produce detectable antibodies to HIV, it is also important to test for the antigen p24. This is often the first positive marker in patients with HIV infection.

Chapter 11

1. b
2. a
3. c

4. d
5. b
6. d
7. a
8. c
9. b
10. d
11. b

Chapter 12

1. d
2. c
3. d
4. d
5. b
6. c
7. a
8. d
9. b
10. d
11. a
12. c
13. b
14. a
15. True

Chapter 13

1. Viridans streptococci are normal microbiota in the throat. Reporting antimicrobial susceptibility test results often suggests to the health care provider that the isolated organism is clinically significant, and antimicrobial therapy should be considered. Reporting antimicrobial susceptibility results on viridans streptococci from the throat may lead to inappropriate use of antimicrobial agents and may also prevent the health care provider from finding the correct cause of the patient's problem.
2. 1.5×10^8
3. Penicillin
4. b
5. c
6. d
7. b
8. a
9. d
10. c
11. a, True; b, false; c, false; d, true; e, false
12. b
13. Endocarditis is a serious infection and is located at a body site in which immune defense mechanisms are not abundant. Therefore it is essential for the antimicrobial agent to kill the bacteria to effect a cure.
14. 99.9%
15. Logarithmic
16. a
17. b
18. c
19. d

Chapter 14

1. *Staphylococcus aureus* is noted for causing suppurative skin infections such as bullous impetigo; furuncles (boils); carbuncles; cellulitis; wound infections associated with trauma, surgery, and burns; pneumonia; organ abscesses; bacteremia; endocarditis; osteomyelitis; and septic arthritis.
2. Although *S. aureus* has had a lengthy association with hospitalized and nursing home patients, recovery in community populations, including pediatric populations and student athletes, has increased.
3. Protein A binds the Fc portion of immunoglobulin G (IgG), thereby interfering with phagocytosis and blocking the protective action of IgG.
4. Toxic shock syndrome (TSS) is associated mainly with TSST-1. However, some cases of TSS have been linked to enterotoxin B or C.
5. Exfoliative toxin or epidermolytic toxin causes staphylococcal scalded skin syndrome.
6. Enterotoxins A to E and G to J, most commonly A and D, are associated with staphylococcal food poisonings.
7. Although not all coagulase-negative staphylococci (CoNS) are considered clinically significant, those CoNS associated with indwelling devices and immunocompromised patients are considered potential pathogens. *S. lugdunensis* infections are usually more invasive. Also, because they have a distinct susceptibility category separate from the other CoNS, it is important to identify suspected *S. lugdunensis* to the species level, as in the case of blood isolates. Urinary tract infections caused by *S. saprophyticus* are also clinically significant.
8. The clinically significant CoNS include *S. epidermidis* and *S. saprophyticus*. In addition, infections (e.g., endocarditis, septicemia, peritonitis) caused by *S. haemolyticus* and *S. lugdunensis* have become more common. *S. saprophyticus* is clinically significant when isolated from urine. Other CoNS, such as *S. pseudintermedius*, will gain importance as they become more frequently recovered and identified using the latest techniques.
9. Tube-coagulase and clumping factor (slide) coagulase are two tests used to detect coagulase activity of *S. aureus*. The tube-coagulase can be used as a confirmatory test for coagulase, whereas the traditional slide coagulase using plasma is considered obsolete.
10. A positive coagulase test would differentiate *S. aureus* from most other staphylococci. Numerous commercial kits contain plasma-coated latex particles or antibodies directed against *S. aureus* molecules that also accurately identify the organism.
11. A disk diffusion test using a 5- μ g novobiocin disk can be used. *S. saprophyticus* will be resistant, whereas most other CoNS will be susceptible.
12. An oxacillin-resistant *S. aureus* isolate is considered resistant to all penicillinase-stable penicillins and most β -lactam antibiotics. Such an isolate is referred to commonly as methicillin-resistant *S. aureus* (MRSA).
13. Those who are at high risk for healthcare-associated MRSA include recently hospitalized patients, especially older adults; people with weakened immune systems; individuals who reside in nursing homes; and patients who have an invasive medical device such as an intravenous line or urinary catheter that can provide a pathway for MRSA to travel into the body. Those who participate in sports or live in crowded or unsanitary conditions are at risk for community-associated MRSA infections. Outbreaks of MRSA have occurred in military training camps, childcare centers, and jails. Carriers of MRSA can spread the bacteria, even if they are not ill themselves.
14. Oxacillin has been used to predict methicillin resistance. However, ceftiofur is now recommended for determining oxacillin resistance in staphylococcal species. For the most accurate detection of methicillin resistance, molecular tests for *mecA* or tests that detect the *mecA* product, PBP2, may be used. For detection of clindamycin resistance, a disk diffusion induction test (D-zone test) of clindamycin and erythromycin should be used. Not all susceptibility methods are able to detect vancomycin-intermediate *S. aureus* (VISA) or vancomycin-resistant *S. aureus* (VRSA). The Clinical and Laboratory Standard Institute and Centers for Disease Control and Prevention have recommended the addition of a vancomycin agar plate, which can be used as a supplemental plate when testing MRSA isolates. Most VISA and VRSA have thus far been detected in these more resistant *S. aureus* strains.
15. Clinical isolates that have been grown on traditional media can be rapidly identified by simple catalase and latex agglutination tests, as described in this chapter. For many laboratories, this will continue to be a cost-effective and efficient way to identify *S. aureus* and CoNS.
16. A Gram stain and catalase test are two initial rapid tests important in determining the presence of staphylococci. Next, a slide latex or hemagglutination assay should be performed. The third-generation tests detect protein A, clumping factor, and capsular polysaccharide serotypes 5 and 8. A positive result indicates *S. aureus*. A negative result suggests a coagulase-negative staphylococci. Laboratories are incorporating molecular tests to identify staphylococci. These methods will provide rapid methods of identification within hours, and they can identify markers of antimicrobial resistance. Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry with databases incorporating commonly recovered species of staphylococci will also become an asset, especially in larger institutions.
17. b
18. a
19. d
20. c

Chapter 15

1. Tests that would be useful in the identification of group A *Streptococcus* (*S. pyogenes*) are PYR (positive), bacitracin susceptibility (sensitive), and SXT susceptibility (resistant) tests, and an immunoassay for detection of the group A antigen.
2. b

3. c
4. d
5. a
6. Penicillin is the drug of choice for the treatment of *S. pyogenes* pharyngitis. Patients who are allergic to penicillin are often treated with erythromycin.
7. *S. pyogenes* has been linked to necrotizing fasciitis. Streptococcal toxic shock syndrome is a systemic disease mediated by a soluble toxin. The infective agent in this case is not necessarily invasive. Rheumatic fever and acute glomerulonephritis are immunologic sequelae and not invasive infections.
8. *S. pneumoniae* is the most common cause of community-acquired pneumonia and of bacterial pneumonia.
9. Group B streptococci (*S. agalactiae*) are rarely associated with disease in healthy adults; however, they are a significant cause of morbidity and mortality in neonates. Neonates can acquire infection in utero following premature rupture of the membrane or during delivery. Pregnant women are often screened for group B streptococci as part of the prenatal workup.
10. Members of the genera *Granulicatella* and *Abiotrophia*, formerly referred to as *nutritionally variant streptococci*, have a requirement for pyridoxal. Isolates are generally able to grow in blood cultures, but agar must be supplemented with pyridoxal to sustain growth on solid media. Alternatively, the bacteria can grow as small pinpoint colonies around colonies of *Staphylococcus aureus*, which secretes small amounts of pyridoxal during growth.
11. Acute glomerulonephritis and rheumatic fever
12. b
13. a
14. c
15. d

Chapter 16

1. b
2. c
3. a
4. d
5. d
6. In the spore stain, vegetative cells are red, and the spores stain green.
7. a
8. c
9. c
10. a
11. d
12. b
13. d
14. b
15. *Streptococcus agalactiae* (group B streptococci) and *Enterococcus* spp. can produce laboratory results similar to those for *Listeria monocytogenes*. Initial differentiation between *L. monocytogenes* and similar microorganisms can be made by the Gram stain, catalase test, and esculin hydrolysis.
16. b
17. c

Chapter 17

1. b
2. a
3. d
4. c
5. b
6. d
7. d
8. c
9. Uncomplicated genital infections with gonorrhea are treated with a single 500-mg intramuscular dose of ceftriaxone. If chlamydial infection cannot be excluded, oral doxycycline (100 mg twice daily for 7 days) should also be administered for coinfection with *C. trachomatis*.
10. d
11. d
12. a
13. a
14. *Moraxella catarrhalis* will grow on SBA and CHOC agar, producing smooth, opaque, gray to white colonies. Identification tests will show that *M. catarrhalis* is oxidase- and catalase-positive. The organism is asaccharolytic, and it may be differentiated from *Neisseria* spp. by positive DNase or butyrate esterase reactions. Several of the commercial multitest systems for the identification of *Neisseria* species also identify *M. catarrhalis* (see Table 17.3).
15. b

Chapter 18

1. *Haemophilus influenzae* will grow only where both X and V factors are present; therefore the bacteria will grow between the two strips where the two factors have diffused and around the XV strip.
2. b
3. a
4. *H. ducreyi* is fastidious and requires enriched media for growth. GC agar supplemented with 1% hemoglobin, 5% fetal calf serum, 1% IsoVitaleX, and 3 mg/L of vancomycin is recommended. The plates must be incubated in an atmosphere of increased humidity and CO₂ (5% to 10%) and at a temperature of about 32° C.
5. Both *H. aegyptius* and *H. influenzae* biogroup aegyptius are noted for causing conjunctivitis. *H. influenzae* biogroup aegyptius, however, is associated with a more invasive disease known as *Brazilian purpuric fever* characterized by conjunctivitis, high fever, vomiting, petechiae, purpura, septicemia, and shock.
6. b
7. d
8. a
9. *Brucella melitensis* will grow on SBA and does not require X or V factor, whereas *H. influenzae* will not grow on SBA and does require X and V factors.
10. *Francisella tularensis* is the most likely causative agent. The primary reservoirs for *F. tularensis* are rabbits. Although some infections are acquired by ingestion,

it is more common to find ulceroglandular infections following direct contact with rabbits.

11. Travel, age (older adults), smoking, alcohol consumption, and an immunocompromised state are risk factors that contribute to severe infections caused by *Legionella* spp.
12. Crowded conditions and warm, humid, environmental sources contribute to infections by *Legionella*. Infections are often linked to contaminated water supplies.
13. Buffered charcoal yeast extract with 0.1% α -ketoglutaric acid (BCYE α) agar is the preferred medium for the recovery of *Legionella* spp.
14. Chlorine tolerance below 2 to 3 mg/L, ability to grow at 20° to 43° C and survive for varying periods at 40° to 60° C, capability to adhere to components of piped water systems, ability to survive in the presence of environmental bacteria and algae, and ability to multiply within free-living protozoa are factors contributing to human infections caused by *Legionella* spp.
15. Urine for the urine antigen test is the best nonrespiratory specimen for the detection of *Legionella* spp.
16. Presumptive identification methods include Gram staining a suspicious colony growing only on BCYE α medium and finding thin, faintly-staining gram-negative rods that may show size variation, from 2 to 20 μ m in length. Also, a test for L-cysteine requirement by subculturing to BCYE α and SBA should be performed. Bacteria demonstrating a requirement for L-cysteine should be typed using anti-*Legionella* antisera.
17. c
18. No, despite vaccination as children, adults are not immune; however, infections in adults are normally mild or asymptomatic. Adults serve as reservoirs for disease in children and adolescents.
19. Nasopharyngeal aspirates and Dacron swabs are the clinical samples of choice for the diagnosis of *B. pertussis* infection.
20. Amies, casamino acid, and Regan-Lowe with charcoal and cephalixin transport media are the most appropriate for the maximum recovery of *B. pertussis*. Direct inoculation of plated media at bedside is preferred over transport media.
21. PCR testing is the most widely accepted method for rapid detection of *B. pertussis*.
22. Both species cause pertussis in children; however, pertussis caused by *B. parapertussis* tends to be milder.
23. No, serologic testing is not widely available, but it can be used as a retrospective epidemiologic tool.
24. c
25. b

Chapter 19

1. Most members of the order Enterobacterales can ferment glucose, are oxidase-negative (except for *Plesiomonas shigelloides*), and are able to reduce nitrate to nitrite (except for *Photobacterium* spp. and *Xenorhabdus* spp.).
2. a, D; b, C; c, A; d, B
3. c
4. a
5. b

6. a
7. d
8. b
9. c
10. d
11. c
12. a
13. a
14. c
15. a

Chapter 20

1. b
2. d
3. a
4. d
5. c
6. a
7. b
8. c
9. Appropriate specimens for the isolation of enteric campylobacters are stool samples and rectal swabs; stool samples are preferred. Two categories of media are available for isolation: blood-based and charcoal-based media. A commonly used blood-based medium is CAMPY-BAP. This is a *Brucella* agar-based medium, with 10% sheep red blood cells and a combination of antimicrobials. Charcoal cefoperazone desoxycholate agar is an alternative. The addition of antimicrobial agents and incubation of the plates at 42° C inhibits normal fecal biota. Because the campylobacters require oxygen at a concentration less than room air, they must be incubated in a microaerophilic atmosphere.
10. The most commonly used nonculture method for the diagnosis of *Helicobacter pylori* is the noninvasive ¹³C-labeled urea breath test. The patient receives an oral dose of labeled urea. Urease activity by *H. pylori* results in the formation of radioactive-labeled CO₂, which is absorbed into the bloodstream and then exhaled. Other nonculture methods include microscopic examination of stained gastric tissue, direct fecal antigen detection, polymerase chain reaction assay, and determining urease activity of gastric biopsy material.

Chapter 21

1. d
2. d
3. c
4. d
5. d
6. c
7. *Pseudomonas aeruginosa*, *Acinetobacter baumannii* complex, *Burkholderia* spp., and *Stenotrophomonas maltophilia* are the four most common nonfermentative, gram-negative bacilli isolated in the clinical laboratory. *P. aeruginosa* is the most common nonfermenter associated with clinical infections, especially healthcare-associated infections. *A. baumannii* complex, *Burkholderia* spp., and *S. maltophilia*

are often isolated from hospitalized patients, especially from respiratory specimens, but they are more often colonizers and are not always clinically significant. The isolation of a nonfermenter from a single blood culture or as part of a polymicrobial infection often indicates that the organism is acting as a colonizer or a laboratory contaminant rather than a relevant pathogen. However, if one of the nonfermenters is seen on a Gram-stained smear from a sterile site, is the only organism isolated, and is present in high numbers, its clinical significance must be considered.

8. Many of the nonfermenters, especially *P. aeruginosa*, *A. baumannii* complex, and *S. maltophilia*, can be resistant to agents used to treat infections caused by fermentative gram-negative bacilli. They are resistant to penicillin, ampicillin, most third-generation cephalosporins (except ceftazidime), macrolides, lincosamides, and agents active against gram-positive bacteria. There is variability of their in vitro and in vivo responses to aminoglycosides; quinolones; aminopenicillins, such as piperacillin and ticarcillin; and sulfamethoxazole. Specific susceptibility tests must be performed if the nonfermenter is considered clinically relevant.

9. d
10. d
11. c
12. b
13. b
14. a
15. c

Chapter 22

1. b, c, d, a, e
2. d
3. c
4. c
5. d
6. a
7. b
8. False
9. False
10. True
11. True
12. True
13. d
14. b
15. a
16. d
17. d
18. b
19. a
20. c

Chapter 23

1. d
2. b
3. a
4. a

5. c
6. d
7. c
8. d
9. d
10. b and d

Chapter 24

1. c
2. d
3. b
4. a
5. d
6. c
7. d
8. c
9. a
10. b
11. The infant in the Case in Point was infected with *Chlamydia trachomatis*. This organism can cause a variety of infections in neonates, including pneumonia and pharyngeal and enteric infections.
12. Lymphogranuloma venereum differs from other diseases caused by *C. trachomatis* because the serovars causing this disease are more invasive. These serovars can survive inside mononuclear cells and are carried into the lymphoid tissue, where they produce a strong inflammatory response.
13. *Chlamydia pneumoniae* is thought to be a common cause of pharyngitis and pneumonia. This organism might be associated with atherosclerosis and coronary heart disease.
14. *Coxiella burnetii* is a facultative intracellular parasite that develops within the phagolysosome of infected cells. *Rickettsia* spp. prevent phagolysosome formation and escape the phagosome and replicate free in the cytoplasm. In addition, although *C. burnetii* is known to infect more than 12 genera of ticks and other arthropods, it is generally not transmitted by arthropods. *C. burnetii*, unlike the *Rickettsia* spp., has been grown in cell-free media.

Chapter 25

1. c
2. a
3. a
4. c
5. d
6. Because of their fastidious nature, routine prenatal cultures would not have detected most mycoplasma. *Mycoplasma hominis* will grow on routine media, but it forms pinpoint colonies after 48 hours of incubation that could be easily missed unless efforts were made to specifically detect this agent.
7. The four species of mollicutes associated with the urogenital tract of humans are *M. hominis*, *M. genitalium*, *Ureaplasma urealyticum*, and *U. parvum*.
8. A7 and A8 are selective and differential media for the isolation of *M. hominis* and *U. urealyticum*. In addition,

Shepard's 10B arginine broth can also be used. *M. hominis* and *M. pneumoniae* can be grown on SP4 broth if arginine is added for the latter. *M. hominis* is the least fastidious of the mollicutes and will grow on sheep blood and chocolate agars.

9. The mollicutes do not cause vaginitis. However, there is evidence that *M. hominis* can contribute to bacterial vaginosis. *M. hominis* has also been isolated from the endometrium and fallopian tubes of women with salpingitis. Meningitis in neonates caused by *M. hominis* and *U. urealyticum* has been reported. Infection could have originated during delivery.
10. Complement fixation assays had been the primary serologic method to detect anti-*M. pneumoniae* antibodies. However, because of technical problems, enzyme immunoassays and immunofluorescent antibody methods are now more commonly used.
11. The mollicutes lack a cell wall and are therefore inherently resistant to the β -lactams: penicillins and cephalosporins.

Chapter 26

1. a
2. c
3. d
4. b
5. c
6. b
7. a
8. c
9. d
10. Many mycobacterial species, saprophytes, and potential pathogens may be isolated from humans. Historically, mycobacteria have been identified by growth characteristics and biochemical testing. More recently, molecular biology assays have been developed. These assays include mycolic acid analysis of bacterial cell walls by high-pressure liquid chromatography, DNA probe technology, DNA sequencing, and MALDI-TOF MS. Genetic probe technology offers tremendous promise in microbial identification at a variety of levels—family, genus, species, and subspecies. The most common probe technology is the commercially available, single-stranded, acridinium ester–labeled DNA probe for the detection of rRNA (Gen-Probe, San Diego, CA). Probes specific for the *M. tuberculosis* complex, *M. kansasii*, and *M. gordonae* and separate probes for *M. avium* and *M. intracellulare* are available. Laboratories should perform identification according to the level of service for which they are qualified. All isolates should be identified to the species level.
11. Slowly growing *M. tuberculosis* has the extraordinary ability to persist and replicate in the harsh environment of the alveolar macrophage. The pathogenic mycobacteria are slow growers, which also lends to drug resistance. Antimicrobial agents are more active against rapidly growing bacteria. The American Thoracic Society, Centers for Disease Control and Prevention, and the International Union Against Tuberculosis and Lung Disease recommend a regimen of rifampin, isoniazid, pyrazinamide, and ethambutol (RIPE treatment) for the first 8 weeks for treatment of tuberculosis. This is followed by isoniazid and rifampin for 18 weeks. Multidrug-resistant tuberculosis, defined as an isolate resistant to at least isoniazid and rifampin, is more complicated to treat.
12. Mycobacteriologic services are available in many laboratories. Clinical laboratory functions that contribute to the diagnosis and management of tuberculosis have been divided into the following three major categories of service offered.
 - Level 1: Collection and transport of specimens and preparation and examination of smears for acid-fast bacilli.
 - Level 2: Procedures of level 1, plus isolation and identification of *M. tuberculosis*.
 - Level 3: All procedures of level 2, plus identification of mycobacteria other than *M. tuberculosis*.
 The determination of drug susceptibility may be performed at level 2 and should be performed at level 3. However, antimicrobial susceptibility testing of the mycobacteria is difficult and should be attempted only by laboratories with experience in this assay. A laboratory may choose to develop or maintain the skills defined under one of the aforementioned levels, depending on the frequency with which specimens are received for isolation of mycobacteria, the nature of the clinical community being served, and the availability of a specialized referral service. All laboratories that perform clinical mycobacteriology should participate in recognized proficiency testing programs, and levels of service should be established and limited by the quality of performance demonstrated in these examinations.
13. Many clinical specimens contain an abundance of nonmycobacterial microorganisms. Unless an attempt is made to inhibit these usually fast-growing contaminants, they can quickly overgrow the more slowly growing mycobacteria. Organic debris (e.g., tissue, serum, other proteinaceous material) surrounding the organism in the specimen must also be liquefied so decontaminating agents will kill undesirable microbes, allowing surviving mycobacteria to gain access to the nutrients in the media. Mycobacteria are slightly more refractory to harsh chemicals; therefore chemical digestion and decontamination procedures have been used with success to enhance the recovery of acid-fast bacteria from clinical specimens. NaOH digests and decontaminates specimens, whereas *N*-acetyl-L-cysteine effectively digests the specimen but does not decontaminate.
14. Because low levels of mycobacteria organism may be present in clinical samples, meticulous care must be practiced during the processing of specimens for the detection and isolation of mycobacterial organisms. False-negative results may occur if processing protocols are not followed appropriately. Prolonged decontamination can have a negative effect by killing the mycobacteria. Improper centrifugation force may also lead to false-negative results. If insufficient force is applied, the mycobacteria may remain in the interface of the processed specimen and can be inadvertently discarded during processing. Care must be applied during culturing of the specimen. False-positive results may occur through carryover from the mouths

of containers used to transfer processing agents, introduction of the organism from environmental sources, such as water, or introduction of organisms from aerosols produced when specimen containers are opened. When making slides, care must be taken to prevent carryover from one slide to another. Slides should never come into contact with one another.

Cross-contamination between specimens may occur if instruments are not properly maintained.

15. The mycobacteria are easily spread by the airborne route; therefore it is important to avoid aerosol-generating procedures. In addition, mycobacterial laboratories should be under negative air pressure so air flows inward when the doors to the laboratory are opened. Specimen processing should be conducted in a biological safety cabinet; laboratory scientists must wear laboratory coats, eye protection, and a respirator (not a surgical mask). When disposable inoculating loops and needles are not used, electric incinerators should be used instead of open flames for sterilizing metal loops and needles. When specimens are centrifuged, they must be in a screw-capped tube in a secondary screw-capped container.

Chapter 27

1. d
2. b
3. b

4. a. *Blastomyces dermatitidis*: Microscopic examination of cultures grown at 35° C reveals large, broad-based budding yeast. At 22° C, the microscopic examination of the mold reveals conidia borne on short lateral branches that are ovoid to dumbbell-shaped and varying in diameter from 2 to 10 µm.
b. *Coccidioides immitis* and *C. posadasii*: Microscopic examination of secretions may reveal spherules containing endospores. At 22° C, microscopic examination of the mold reveals alternating (separated by a disjunct cell) hyaline arthroconidia.
c. *Histoplasma capsulatum*: Microscopic examination of cultures grown at 35° C reveals small yeast cells. The yeast cells measure 2 to 3 µm by 4 to 5 µm. At 22° C, microscopic examination of the mold reveals nondescript spherical microconidia and characteristic tuberculate macroconidia.
d. *Sporothrix schenckii*: Microscopic examination of cultures grown at 35° C reveals small, cigar-shaped yeast cells. Microscopic examination of the mold phase reveals conidia developing in a rosette pattern at the ends of delicate conidiophores in addition to dark-walled conidia being produced along the sides of the hyphae.
5. a. *Nannizzia gypsea*: Microscopic examination reveals fusiform, moderately thick-walled conidia measuring 8 to 15 µm by 25 to 60 µm, with as many as six cells. In some isolates, the distal end of the macroconidium may bear a thin, filamentous tail.
b. *Microsporium canis*: Microscopic examination reveals spindle-shaped macroconidia with echinulate, thick walls measuring 12 to 25 µm by 35 to 110 µm with 3 to 15 cells. The tapering, sometimes elongated, spiny distal ends of macroconidia are key features that distinguish this species.
c. *Trichophyton rubrum*: Microscopic examination reveals clavate or peg-shaped microconidia formed along undifferentiated hyphae. Rare macroconidia may be formed.
d. *Trichophyton mentagrophytes*: Globose microconidia are found primarily in clusters described as grapelike or engrape. Macroconidia are thin-walled, smooth, and cigar-shaped, with four to five cells separated by parallel cross walls.
6. *Trichophyton rubrum* is urease-positive, whereas *T. mentagrophytes* is typically urease-negative. Wedge-shaped perforations in the hair shaft (positive) are characteristic of *T. mentagrophytes* as well as *M. canis* and *M. gypseum*. Species negative for hair perforation include *T. rubrum*, *M. audouinii*, and *M. praecox*.
7. a
8. Saprobies are usually environmental contaminants and are not considered human pathogens. However, saprobies may cause disease in individuals with impaired immune systems. When a saprobe is recovered from an immunocompromised patient, the laboratory should work closely with the physician to help determine if the isolate is an environmental contaminant, or if it is possibly causing disease.
9. *Penicillium* spp. and *Aspergillus fumigatus* can form blue-green colonies, but they may be differentiated microscopically in that conidia in the aspergilli are formed from phialides attached to a vesicle or metula, whereas conidia in the genus *Penicillium* are formed from branched conidiophores bearing phialides at their tips. *Fusarium* spp. are typically white to pink to purple colonies that produce large, hyaline, thin canoe- to green bean-shaped macroconidia. *Curvularia* spp. have black colonies that produce wide, phaeoid (dematiaceous), crescent to half circle-shaped conidia.
10. Lesions of chromoblastomycosis most often appear as verrucous nodules that may become ulcerated and crusted. Long-standing lesions have a cauliflower-like surface. Brown, round, sclerotic bodies, which are nonbudding structures occurring singly or in clusters, are seen in tissues. These sclerotic bodies reproduce by dividing in various planes, resulting in multicellular forms. Lesions of mycetoma are characterized by tumefaction, draining sinuses, and granules. Mycetomas are localized infections that involve the cutaneous and subcutaneous tissue, and possibly bone. Granules are released through the draining sinus tracts.
11. Fungi are eukaryotic and possess a true nucleus, with a nuclear membrane and organelles like mitochondria. Bacteria are prokaryotic and lack these structures. Unlike plants, fungi lack chlorophyll and do not undergo photosynthesis and must absorb nutrients from the environment. In addition, fungal cell walls are made of chitin, whereas those of plants contain cellulose. Bacteria lack chitin and cellulose.
12. Fungal colonies, although occurring in many colors, may be generally divided as hyaline or phaeoid. Hyaline molds are light-colored, whereas phaeoid fungi are darkly pigmented. This pigmentation is a result of

melanin in the cell wall of the organism. The Fontana-Masson stain will detect phaeoid hyphae in the tissues because it stains melanin.

13. Teleomorph is the sexual stage of a fungus, whereas the anamorph is the stage that reproduces asexually. In certain species, more than one asexual stage is associated with the same teleomorph. When more than one anamorph exists, they are termed *synanamorphs*.
14. The germ tube test would be positive within 2.5 to 3 hours, and the cornmeal agar would result in true hyphae and pseudohyphae, with rare chlamydoconidia.
15. c
16. c
17. b
18. c
19. a
20. c

Chapter 28

1. The most likely identification is *Giardia duodenalis*; these parasites are pathogenic for humans.
2. Both thick and thin smears are routinely performed on blood specimens to detect parasites. Because the thick smear is made with a larger volume of blood, it is more sensitive than a thin smear. However, species identification is made from the thin smear because the red blood cells are intact and parasites have a more characteristic morphology.
3. The eggs of *Taenia* spp. are round, are approximately 35 μm in diameter, have a striated border, and contain a hexacanth oncosphere. The eggs of *T. solium* and *T. saginata* cannot be differentiated. *A. lumbricoides* eggs are fertilized or unfertilized. The fertilized eggs are slightly oval, with a thick mammillated coat, and they measure about 75 μm $\times$ 50 μm . The unfertilized eggs are more oval and are as long as 90 μm , with a thick mammillated coat or an extremely minimal mammillated layer. Eggs of *T. trichiura* are oval with plugs on both poles; they are about 52 μm $\times$ 22 μm . Hookworm eggs are oval with broad ends, have a clear space between the thin shell and embryo, and measure 55 to 75 μm $\times$ 35 to 40 μm . The eggs of the three human hookworm species, *Ancylostoma duodenale*, *Necator americanus*, and *Ancylostoma ceyanicum* cannot be differentiated.
4. The gravid females of *E. vermicularis* (pinworm) migrate out of the anus to deposit eggs; therefore stool samples are not the specimen of choice. The best method for detection of pinworm eggs is to use the scotch tape or sticky paddle technique.
5. *C. cayetanensis* oocysts are round and average 8 to 10 μm in diameter. On a wet mount, they appear nonrefractile, with a cluster of granules. The organisms are not readily distinguishable on trichrome-stained smears and may appear as round, wrinkled, clear objects. With modified acid-fast stain, the organisms stain variably from dark pink to almost colorless and may have a wrinkled or distorted shape. Oocysts of *Cyclospora* autofluoresce blue under ultraviolet light, at a wavelength of 365 nm, and green at 450 nm. *C. parvum* oocysts are round and approximately 4 to 6 μm in diameter. Because of their small size, they are not distinguishable on wet mounts or in most permanent-stained smears. With a modified acid-fast stain, they are dark pink and round. Direct fluorescent antibody techniques that detect the oocyst in the feces are available.
6. The most likely identification is *Entamoeba histolytica* trophozoite. If no ingested erythrocytes had been seen, the organism would have been reported as *E. histolytica*-*E. dispar* because the organisms are morphologically identical. The nonpathogenic *Entamoeba moshkovskii* and *Entamoeba bangladeshi* are also morphologically indistinguishable from *E. histolytica*. The presence of ingested erythrocytes indicates that it is *E. histolytica*. This parasite is considered pathogenic for humans. Patients with *E. histolytica* infection can suffer from amebic dysentery, which can manifest as fever and intense colicky abdominal pain.
7. *C. parvum* produces two types of oocysts, thin-walled and thick-walled. The thick-walled oocysts are passed in the feces and are infective. The thin-walled oocysts are involved in autoinfection. These structures rupture in the intestine and release sporozoites that reinfect intestinal cells. With *S. stercoralis* infection, the eggs rupture in the intestine and release rhabditiform larva that mature to filariform larva in the intestine. The filariform larvae burrow into the intestinal wall, gain access to the circulation, and then complete the life cycle. Autoinfections result in persistent infections with large numbers of parasites.
8. The organism belongs to the genus *Trypanosoma*. The morphologic form is the trypomastigote. Members of the genus *Trypanosoma* cause African (trypanosomiasis) sleeping sickness, transmitted by tsetse flies, and Chagas disease, transmitted by kissing or reduviid bugs.
9. Primary amebic meningoencephalitis is caused by *Naegleria fowleri*. Young, healthy individuals are usually infected and present with a history of swimming in warm, stagnant water. The trophozoite gains direct access to the brain via the nasal olfactory nerve when water is forced into the nose. The infection has a rapid onset (within 2 to 7 days) after exposure, and symptoms resemble those of bacterial meningitis—photophobia, headache, vomiting, and stiff neck. Death usually occurs within 1 week of onset of symptoms. Diagnosis can be made by observing the ameboid trophozoite in a wet mount of the CSF sediment. Trophozoites may be found in biopsy tissue of the brain. Granulomatous amebic encephalitis is a chronic infection caused by *Acanthamoeba* spp. and *Balamuthia mandrillaris* and is usually found in individuals with underlying conditions such as lymphoma or diabetes. The organism may enter through the skin or be inhaled, and it spreads hematogenously to the brain. It may take years for symptoms to develop. Symptoms include headache, dizziness, mental confusion, and seizures. Often, the infection is detected at autopsy. Trophozoite and cyst forms can be identified on biopsy specimens.
10. Toxoplasmosis in the immunocompetent host is usually asymptomatic or presents as a transient, flulike illness. The immunocompromised patient often develops encephalitis or pneumonia. *Cryptosporidium* infection in the immunocompetent host causes self-limiting diarrhea. In the immunocompromised host,

the diarrhea is prolonged and severe (partially because of the autoinfective part of the life cycle). The patient may lose as much as 10 L/day of fluid and develop electrolyte imbalance. Long-term infection may lead to malabsorption syndrome. *Strongyloides* infection in the immunocompetent host also may be asymptomatic or present with vague abdominal symptoms that can mimic those of an ulcer. In the immunocompromised host, hyperinfection (dissemination) may result, with filariform larvae migrating to multiple body organs.

11. b
12. c
13. a
14. a
15. b
16. c
17. d
18. c
19. b
20. c

Chapter 29

1. c
2. a
3. b
4. d
5. a
6. c
7. d
8. a
9. b
10. c
11. Some opportunistic infections and conditions associated with AIDS include esophageal candidiasis, cryptococcal meningitis, cryptosporidiosis, histoplasmosis, persistent HSV infections, mycobacterial infections, *Pneumocystis* pneumonia, and Kaposi sarcoma.
12. Testing for HIV-specific antigens and antibodies is important in the diagnosis of HIV infection, including antibodies to viral antigens p24, p31, gp41, and gp120/160 and HIV p24 antigen.
13. EBV causes infectious mononucleosis. EBV has been associated with Burkitt lymphoma, nasopharyngeal carcinoma, and the development of B-cell lymphoproliferative disorder or lymphoma. Other EBV complications include splenic hemorrhage, hepatitis, thrombocytopenia purpura with hemolytic anemia, Reye syndrome, and encephalitis.
14. Signs and symptoms of acute HBV infection include fever, anorexia, and hepatic tenderness, with jaundice occurring in 30% to 50% of infected older children and adults. The immune response slowly clears HBV from the body, and most patients become noninfectious. Some patients become HBV carriers for longer than 6 months, and these patients are very likely to carry the virus for much longer. Chronic hepatitis patients have a higher risk of cirrhosis or hepatic carcinoma. Acute hepatitis resolves as HBsAg clears, and anti-HBs can be detected. As infection resolves, HBeAg fades, and anti-HBe appears. In chronic hepatitis patients, HBsAg persists,

and the corresponding antibody cannot be detected. Similarly, HBeAg can persist.

15. Dengue fever (DF) is a fairly mild, self-limiting disease, with patients experiencing fever, headache, myalgia, and bone pain. Dengue hemorrhagic fever is a serious infection, and these patients develop the symptoms of classic DF along with thrombocytopenia, hemorrhage, and shock. Death can result from the infection.
16. Fluorescent antibody and ELISA methods are available for the rapid detection of rabies antigen in brain tissue and are the preferred methods. A classic method for the diagnosis of rabies was to detect Negri bodies, eosinophilic cytoplasmic inclusions, in neurons. This test is insensitive and no longer recommended.
17. Many human papillomaviruses (HPVs) cause plantar, common, and genital warts. Some strains are associated with a rare autosomal disease called *epidermodysplasia verruciformis*, and some are associated with cervical cancer. A vaccine (Gardasil-9) is available for some of the strains associated with cervical cancer.
18. The viruses most noted for latency are the herpesviruses.
19. Influenza viruses mutate often because of replication errors. These mutations cause antigenic drift, ensuring antigenic variability of strains each year. Recombination events of the influenza A genome result in a major antigenic change called *antigenic shift*. Health care agencies predict the most likely strains that will predominate in the next season. Quadrivalent influenza vaccines are available before the start of the influenza season. Although this process is successful, occasionally an unexpected strain will predominate, and the vaccine may not provide total coverage for that strain. Even with this known possibility, it is still advised that all persons be vaccinated because some protection is better than no protection.
20. Messenger RNA (mRNA) vaccines have provided the best immunity against coronavirus 2, the cause of coronavirus disease 2019. The vaccine contains mRNA that directs the production of a viral spike protein. Antibody to the spike protein is protective.

Chapter 30

1. c
2. d
3. d
4. b
5. d
6. a
7. c
8. c
9. a
10. a
11. b
12. d
13. Advances in scientific technology and greater understanding of the virulence mechanisms of *Bacillus anthracis* have led to the ability to weaponize this organism, making it much easier to disseminate throughout the population as opposed to natural means of infection. Smallpox was declared eradicated in 1979,

so very few individuals since then have been vaccinated. The known threat of existing smallpox strains that could be released into such a naive population could affect millions of people and devastate national health care infrastructures.

14. LRN reference laboratories use standardized reagents and controls and agent-specific protocols for the confirmatory identification of several biothreat agents. They are the laboratories that respond to a sentinel laboratory request for identification.
15. Tier 1 agents are considered to be the highest risk to the public and national security because of their ease in dissemination and transmission and known high morbidity and mortality rates, aimed to cause public panic and social disruption. Agents in the non-Tier 1 group have lower mortality rates and are moderately easy to produce and disseminate.
16. a. *Bacillus anthracis* grown on sheep blood agar plates are characterized as large (2 to 5 mm), flat, irregularly round colonies with a wavy border (Medusa head) and a ground-glass appearance. Colonies are nonhemolytic and are tenacious (stiff egg whites). Gram-stained smear shows large, gram-positive rods. The organism is nonmotile and catalase-positive.
- b. *Yersinia pestis* is a slow-growing organism and will appear pinpoint in size on sheep blood agar or MacConkey agar plates. Usually, 48 hours of incubation is required to identify the organism. It is a nonlactose fermenter. Gram-stained smear may show small, bipolar, gram-negative rods (safety pin appearance). Essential biochemical reactions are oxidase negative, catalase positive, indole negative, and urease negative.
- c. *Francisella tularensis* is a slow-growing organism that must be incubated up to 48 hours on primary plates to produce visible growth. This organism has a requirement for cysteine and thus will not grow very well on sheep blood agar. Chocolate agar or a similar medium will allow growth. Colonies appear pinpoint grayish white after 48 hours. It is a faintly staining, gram-negative coccobacillus. Essential biochemical reactions are oxidase negative, catalase weak positive, β -lactamase positive, satellite test negative, and urease negative.
- d. *Burkholderia* spp. are very slow-growing organisms, requiring up to 72 hours to form mature colonies. They are gram-negative rods. Key biochemical reactions include oxidase variable, catalase positive, indole negative, and resistance to colistin.
17. a. For Ebola virus and Marburg virus, specimens include serum, heparinized plasma, or whole blood collected during the acute febrile stages of illness. Additionally, spleen, lymph nodes, liver, and kidney obtained at autopsy may also be submitted.
- b. To detect *Clostridium botulinum* toxin, specimens include feces, enema fluid, gastric aspirates or vomitus, tissue or exudates, and postmortem specimens.
- c. For suspected anthrax exposure, specimens to be submitted depend on the type of exposure and, thus, sample should be collected from the primary site of infection. Cutaneous anthrax would require specimens from the skin; vesicular fluid from fresh vesicles or material from under the edge of the eschar

are necessary. Gastrointestinal anthrax requires abdominal ascites or specimens from stool or rectal swabs. Blood is the best specimen for inhalational anthrax.

- d. Variola virus specimens are highly infectious and should be treated with BSL4 precautions. Variola virus specimens include vesicle, pustule, scab, or fluid from the patient's pox.

Chapter 31

1. b
2. d
3. c
4. b
5. a
6. c
7. a
8. d
9. c
10. a
11. The first stage in biofilm formation is attachment to a solid surface. This stage is irreversible and is mediated by attachment of molecules and pili. The next stage is characterized by the formation of small cell aggregates and loss of motility. Once the biofilm contains multilayers of cells and has reached a thickness of about 10 μ m, it is in stage III. When the biofilm reaches its maximum thickness, generally about 100 μ m, it is in stage IV. The fifth and final stage begins when cells are dispersed from the biofilm. These cells can presumably establish a biofilm downstream.
12. Biofilms aid microbial attachment because of the production of exopolysaccharide (EPS) by some members of the community and cell-to-cell interactions, resulting in the formation of cell aggregates. The EPS can bind to bacteria of many different species leading to cell aggregate formation.
13. A mature multispecies biofilm will consist of many microcolonies containing different species of microorganisms in several layers. The microcolonies are described as being mushroom-shaped and anchored to the substratum. The microcolonies are surrounded by a matrix consisting of EPS, DNA, and other molecules. Flowing around the microcolonies are water channels that can carry nutrients, DNA, and bacteria.
14. Biofilms can increase antimicrobial tolerance by acting as barriers to penetration. Nutrients, antimicrobial agents, and other molecules may be trapped by the matrix and diffuse slowly; they may not even reach the cells in the lower layers. In addition, because the bacteria near the substratum have fewer nutrients and may be in an anaerobic environment, they are growing slowly, and slow-growing microorganisms are more resistant to antimicrobial agents. Some members of the biofilm can secrete molecules, like penicillinase, that inactivate antimicrobial drugs. Finally, the environment within a biofilm facilitates the exchange of genetic material, and drug-resistant genes can be readily transferred from one organism to another.
15. Virulence mechanisms of biofilms include aiding microbial attachment, increasing the efficiency of

community metabolism, enhancing antimicrobial drug tolerance, protecting against host defenses, facilitating gene transfer, stimulating an inflammatory reaction that damages host tissue, and allowing the spread of microorganisms to other body sites.

16. Indwelling medical devices increase the risk of infection by providing a substrate for microbial attachment. Many microorganisms can readily adhere to hydrophobic plastics and polymers, inducing the formation of a biofilm. Once a biofilm is established, cells can break off and establish an infection downstream.
 17. Areas of concern for clinical microbiologists when dealing with biofilms include false-negative cultures, viable but nonculturable organisms, underestimated colony counts, inappropriate specimen collection, and increased antimicrobial resistance.
 18. One method for studying potential biofilm formation in vitro is based on the staining of microorganisms attached to a solid surface, such as wells of a microtiter plate or the inside of test tubes. The color intensity of the stained cells can be read by a spectrophotometer. Alternatively, bacteria can be allowed to grow in the presence of silicone disks. Growth on the disk is measured by a colorimetric method.
 19. When a biofilm in an individual is disrupted by treatment, bacteria can be released. These bacteria can establish an infection elsewhere in the host. It is important for health care providers to monitor patients after treatment for such an occurrence.
 20. Monoclonal antibodies directed against DNABII proteins might disrupt biofilms. The DNABII proteins are positioned at the vertices of the cross strands of extracellular DNA within the biofilm. Disruption of these proteins results in destabilization of the biofilm causing it to disaggregate.
5. Transmission of SARS-CoV-2 is thought to be primarily through exposure to large droplets, but airborne transmission has also been recognized. Unlike influenza, it was subsequently learned that many infected individuals are asymptomatic, and both asymptomatic and presymptomatic transmission are significant contributors to the spread of infection, making containment particularly challenging.
 6. The common bacterial pathogens that cause community-acquired pneumonia are *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis*, and, increasingly, *Staphylococcus aureus*.
 7. Influenza viruses are constantly changing genetically. The changes can evolve slowly in the population due to mutations caused by the error-prone viral RNA polymerase. This process is termed *antigenic drift*. It is associated with influenza of the same HN phenotype (e.g., H3N2) that has subtle changes in these viral antigens, which can render existing host immunity less effective. In contrast, *antigenic shift* is a term used for a relatively sudden appearance in the global population of an influenza strain that has resulted from genetic recombination (so-called *gene segment reassortment*) among multiple viruses. Whereas genetic drift occurs almost annually, genetic shift is an uncommon epidemiologic event, having occurred only a few times since the 1800s. Antigenic shifts are unpredictable and have the major problem of a much higher transmission rate because of the lack of immunity to these new viruses by most people in the global population. Influenza A can undergo both types of genetic change, whereas influenza B changes mostly by genetic drift.
 8. There is a difference between the groups of pathogens that cause community-acquired and healthcare-associated pneumonias, which makes diagnosis and management different for pneumonias in these two settings. For example, community-acquired pneumonia is more likely to be caused by streptococci, whereas healthcare-associated pneumonia is more likely to be caused by gram-negative bacilli, such as *Pseudomonas*, *Klebsiella*, and *Enterobacter*. The pathogens that cause healthcare-associated pneumonias are more likely to have increased resistance to antimicrobial agents and thus be more difficult to treat.
 9. The absolute CD4 cell count is a surrogate marker for assessing the level of immunosuppression and the risk of HIV-associated complications. When CD4 cell counts are relatively preserved, there is generally no increased risk of opportunistic infections, although these patients remain at increased risk of virulent respiratory infections that also affect the immunocompetent individuals, including tuberculosis and pneumococcal pneumonia. When the CD4⁺ cell count falls, the risk of infection with opportunistic pathogens such as *Pneumocystis jirovecii* increases significantly.
 10. In patients such as the one described here, tuberculosis (TB) should be high on the list of differential diagnoses. Because TB can be a presenting illness in HIV-infected patients, all patients newly diagnosed with active TB should also be tested for HIV infection.
 11. *Bacillus anthracis*, *Yersinia pestis*, and *Francisella tularensis* have been identified as bacterial pathogens that might

Chapter 32

1. The normal microbiota of the respiratory tract plays an important role in protecting the host from infection. Isolation of normal biota from a microbiologic specimen will have less clinical significance than the isolation of pathogenic microorganisms capable of causing disease.
2. Immunocompromised hosts are at risk to develop infection with opportunistic pathogens. These organisms are of low virulence and generally are not pathogenic in hosts with an intact immune system.
3. Opportunistic pathogens are so-named because they generally do not cause disease in normal hosts but require the “opportunity” of an impairment of host defense mechanisms to cause disease.
4. Certain pathogens exhibit seasonal variation and are more common during certain times of the year. Awareness of seasonal trends helps narrow the possible causes of infection, thereby facilitating a more accurate and timely diagnosis. This trend is seen in winter, when there is an increase in viral infections, such as influenza and related postviral bacterial pneumonias, such as those caused by *Streptococcus pneumoniae* and *Staphylococcus aureus*.

be used as agents of bioterrorism and clinically present as severe respiratory infections.

12. The diagnostic microbiology laboratory must be alerted if these agents are suspected in the diagnosis of pneumonia because culture isolation of these microorganisms poses a risk to laboratory personnel and must be performed under conditions of biocontainment.
13. c
14. d
15. b
16. a
17. c
18. a
19. c
20. b

Chapter 33

1. *Candida* spp. can be part of the normal skin microbiota and can enter the bloodstream when intravenous catheters are in place by adhering to and forming a biofilm on the catheter. In addition, total parenteral nutrition has been associated with the development of candidemia; glucose solutions and lipid emulsions can enhance *Candida* biofilm formation.
2. Folliculitis is an inflammation and infection of hair follicles often caused by *Staphylococcus aureus*. Folliculitis can progress to involve deeper tissues and result in nodules called furuncles, which are red, firm, and can drain pus. Carbuncles are lesions that extend even deeper, into the subcutaneous fat, and are composed of multiple abscesses that coalesce. Carbuncles can be associated with systemic symptoms like fever and bacteremia.
3. Cellulitis in diabetics is often caused by staphylococci or streptococci. Ulcerative lesions and gangrene may also be caused by staphylococci and streptococci but are often mixed infections, including gram-positives, members of the order Enterobacterales, *Pseudomonas*, and anaerobic bacteria including *Clostridium* spp.
4. Zoonotic diseases are those transmitted to humans by wild or domestic animals. There are hundreds of zoonoses, but some of the more common ones that cause skin and soft tissue infection include Rocky Mountain spotted fever (caused by *Rickettsia rickettsii* and transmitted by ticks), leptospirosis, bartonellosis, and tularemia.
5. The most common molds causing skin lesions in this setting are *Fusarium* and *Aspergillus*, opportunistic fungal pathogens that infect immunocompromised persons. They invade blood vessels and cause tissue necrosis. *Fusarium* can be isolated in blood cultures, but *Aspergillus* is only rarely detected this way.
6. Members of the family Herpesviridae are characterized by the establishment of lifelong infection. The initial infection is often accompanied by symptoms, but this is usually followed by a prolonged asymptomatic period, during which the virus latently infects the host. The herpesviruses that are most likely to reactivate in adulthood after a period of latency include varicella-zoster, leading to shingles, and herpes simplex virus, leading to outbreaks of oral or genital ulceration.
7. Swimmer's itch is a very pruritic, papular rash caused by bird or animal schistosomes. They penetrate the skin of humans who bathe or swim in infected waters. These nonhuman schistosomes do not mature in humans, and they die in the skin. *Creeping eruption* or *ground itch* are common terms for the dermatitis known as *cutaneous larva migrans* (CLM). The CLM syndrome is caused by the subcutaneous migration of larvae of animal nematodes such as dog and cat hookworms, *Ancylostoma caninum* and *Ancylostoma braziliense*, respectively. The larvae of these and other worms can penetrate the skin of humans and produce a self-limited, itchy, indurated dermatitis.
8. *Staphylococcus aureus* may produce toxins that cause the exfoliating skin condition known as *staphylococcal scalded skin syndrome*, and toxin production by *S. aureus* or *Streptococcus pyogenes* may produce a diffuse erythroderma associated with toxic shock syndrome. Toxin production by *S. pyogenes* may also result in scarlet fever, characterized by a red, sandpaper-textured rash.
9. Infections caused by *Actinomyces* and *Nocardia* can be characterized by the formation of so-called *sulfur granules*, yellow particles consisting of masses of tangled filamentous bacteria. Microscopic analysis of the granules by staining and by culture can identify the infecting organism.
10. It is important to collect the proper specimen at the correct time in the course of the infectious process. The skin surface should be cleansed to reduce the likelihood of recovering colonizing microbiota not associated with the infection. It is recommended to collect samples from the outer edge of larger lesions. The specimen must be maintained under conditions that preserve the viability of the pathogens. This often includes the use of a transport medium appropriate for the expected pathogens (i.e., bacterial, fungal, or viral).
11. a
12. b
13. d
14. c
15. a
16. c
17. a
18. d
19. b
20. a

Chapter 34

1. Gastrointestinal tract host defense mechanisms help prevent pathogens from causing diarrheal illness. The stomach has an acidic environment, with a pH less than 4, which kills more than 99.9% of coliform bacteria within 30 minutes. The small intestine prevents infection through peristalsis. The constant motion prevents bacteria from adhering to the intestinal wall and thus prevents the bacteria from causing infection. The colon and small intestine prevent infection through their normal biota—resident bacteria that normally live in these sites. The normal biota competes with pathogens for nutrients and can also produce toxins to kill pathogenic bacteria. The colon also produces IgA antibody to help fight pathogens.

2. A detailed history and physical examination is often all that is needed to manage acute diarrheal illness. The history should help identify the cause of illness. It should include a detailed dietary history of the 4 to 5 days before symptom onset; contact with individuals with similar symptoms; travel history; recreational activities such as hiking, backpacking, and swimming; and recent antimicrobial use. The symptoms should be well characterized through questions about duration, inflammation, history of previous gastrointestinal symptoms, and any other associated symptoms. One should also ask about other medical illnesses, specifically HIV and cancers, and about all medications that the patient is taking. All this information can help identify the possible causes of diarrhea.
3. Laboratory studies are often not indicated for acute diarrhea because most episodes resolve within 1 day. However, laboratory testing is indicated for those with persistent diarrhea, severe illness, or suspicion of an outbreak. Tests that are usually done include the following: (1) white blood cell count to assess for invasive infection; (2) hemoglobin—anemia can be present with blood loss or hemolytic infection; (3) platelet count; (4) electrolyte panel, which assesses for abnormalities in sodium, potassium, bicarbonate, and creatinine levels as an indicator of hydration status; and (5) fecal leukocyte count to look for evidence of inflammation. If a bacterial infection is suspected, a stool culture is sometimes performed to identify the pathogen.
4. Cytomegalovirus (CMV) is the primary virus that causes diarrhea, often associated with pain, bloody stools, and fever, in immunocompromised patients.
5. An immunocompromised condition can allow a wider range of infectious agents to produce diarrhea. Besides the agents typically found in immunocompetent individuals (bacteria and parasites), the laboratory should screen for mycobacteria, cytomegalovirus, histoplasmosis, and *Strongyloides*.
6. *Entamoeba histolytica* causes fever and grossly bloody diarrhea; however, if the disease is mild, it also can present as watery diarrhea. The trophozoites of *E. histolytica* can leave the intestines and cause metastatic illness, such as a liver abscess.
7. Scombroid is a toxin-mediated illness associated with fish exposure, usually tuna, mackerel, and yellow jack. The tissues of the fish contain histamine and enzyme inhibitors, which cause the symptoms. Symptoms are rapid in onset and include flushing, headache, crampy abdominal pain, and diarrhea. Ciguatera is caused by ciguatera toxin, which is produced by dinoflagellates. Cases of illness are associated with snapper, sea bass, grouper, and barracuda exposure. Symptoms include diarrhea, abdominal pain, weakness, paresthesia, and headache. Other toxin-mediated illnesses associated with fish include paralytic shellfish poisoning (resembling ciguatera) and tetrodotoxin, found in puffer fish, which results in death in more than 50% of patients exposed.
8. Most bacterial diarrheal infections are mild and easily treated with antimicrobial agents; however, rarely, complications can occur. For example, Guillain-Barré syndrome, an ascending paralysis, has been associated with *Campylobacter jejuni* infection. Hemolytic-uremic syndrome is associated with infection with enterohemorrhagic *Escherichia coli* (EHEC) and *Shigella* spp. and is characterized by hemolytic anemia, low platelet count, and kidney failure. Any severe illness can result in death if supportive care is not available; for example, cholera leads to death from dehydration in many developing countries.
9. *Clostridioides difficile* infection is associated with prior antimicrobial use. The use of antimicrobials can suppress the growth of the resident microbiota, favoring the growth of drug-resistant *C. difficile*.
10. *Vibrio cholera* serogroups O1 and O139 have been implicated as causes of epidemic cholera.
11. Traveler's diarrhea is a common problem in patients visiting developing countries; most cases are mild and self-limiting. The most common bacterial cause is enterotoxigenic *Escherichia coli* (ETEC); rotavirus is the most common viral pathogen. Traveler's diarrhea is best avoided by drinking only bottled beverages and avoiding high-risk foods and ice. High-risk foods include any foods prepared by another person and served uncooked (e.g., salad, fruits, vegetables), dips and other foods left standing at room temperature, and raw or partially cooked fish or shellfish.
12. c
13. b
14. a
15. b
16. d
17. b
18. a
19. c
20. c

Chapter 35

1. The cerebrospinal fluid (CSF) is produced by filtration and secretion from specialized capillary tufts of the choroid plexus within the four ventricles of the brain. The CSF flows from the two lateral ventricles to the third ventricle and enters the fourth ventricle via the aqueduct of Sylvius. From there, the CSF enters the basal cisterns and circulates over the cerebellum and convexities of the cerebral hemispheres. The CSF is absorbed primarily by the arachnoid villi through tight junctions of their endothelium.
2. CSF is a clear, colorless, and sterile fluid. In healthy adults, the protein level is 20 to 60 mg/dL, and the glucose level is 40 to 80 mg/dL (CSF glucose-to-serum glucose ratio of 0.6). Normally, few white blood cells are present.
3. The common bacteria causing acute meningitis include *Streptococcus pneumoniae*, *Neisseria meningitidis*, *Streptococcus agalactiae* (group B *Streptococcus*), *Haemophilus influenzae*, and *Listeria monocytogenes*. Patients with sickle cell anemia, splenectomy or asplenia, malignancy, malnutrition, and chronic renal or liver disease are more likely to develop serious *S. pneumoniae* infection. Individuals who are deficient in terminal components of complement (C5 to C9) or

properdin are at higher risk for *N. meningitidis* infection. *S. agalactiae* is a common cause of meningitis in neonates and infants up to 3 months of age. *Escherichia coli* is a less common cause of infection in this population. Neonatal acquisition usually results from vertical transmission from mother to infant. Before the widespread use of a conjugated vaccine, *H. influenzae* was a common cause of invasive illness in young children. The incidence of invasive disease by this organism has been substantially reduced. Infection caused by *L. monocytogenes* is more commonly seen in neonates, older patients, alcoholics, and immunosuppressed individuals.

4. In acute bacterial meningitis, the CSF is turbid because protein levels and white blood cell counts are significantly raised. Primarily, the neutrophils are increased in number. Glucose levels are very low (<40% of the serum glucose concentration) in most patients with bacterial meningitis. In viral infections, the number of lymphocytes is increased in the CSF, and protein concentrations are slightly elevated, whereas the glucose concentration is near the reference range. Fungal and tuberculous meningitis is characterized by increased lymphocytes and elevated protein and decreased glucose levels.
5. *Candida* spp., *Aspergillus* spp., *Mucor*, *Rhizopus*, and *Absidia* are the common fungi that can cause brain abscesses. *Blastomyces dermatitidis* is also associated with abscesses.
6. *Cryptococcus neoformans/gattii*, *Coccidioides immitis*, *Histoplasma capsulatum*, *Blastomyces dermatitidis*, and *Candida* spp. are fungal agents associated with meningitis.
7. CSF specimens should be examined as soon as they are received because some of the infectious agents are fastidious and can become nonviable if specimens are not processed quickly. In addition, some infectious agents found in CSF can be diagnosed by rapid assays, facilitating proper treatment of life-threatening infection.
8. Initial examination of a CSF specimen includes a macroscopic description (i.e., turbidity) and microscopic examination of Gram-stained smear. Many laboratories will also perform an antigen detection assay for *C. neoformans*. Antigen detection assays for bacterial meningitis have low sensitivities and are generally not recommended. Nucleic acid amplification tests have been developed for many bacterial agents and have demonstrated improved sensitivity.
9. Sheep blood and chocolate agar media incubated in 3% to 5% CO₂ at 35° C are generally used for bacterial culture of CSF.
10. Tests performed on CSF for the diagnosis of infectious agents include blood chemistry (protein and glucose), cell counts and white blood cell differential, direct antigen detection for *Cryptococcus*, polymerase chain reaction assays, and cultures for bacteria, fungi, and occasionally viruses. In addition, it is sometimes relevant to test for antibodies to common pathogens found in the central nervous system.
11. c.
12. c
13. a
14. d
15. b

Chapter 36

1. The patient had an indwelling catheter, which was likely the source of infection. Because it was an endovascular source, however, it is considered a primary bacteremia.
2. The patient has granulocytopenia, which placed him at risk for all types of bacterial infections—in particular, bacteremia. Gram-positive organisms are currently the most common causes of bacteremias in the United States.
3. The following conditions also place patients at an increased risk for bacteremia: reduced immune competency, increased use of invasive procedures and instrumentation, very young or very old age, trauma, and administration of immunosuppressive therapy and other drugs.
4. Sources of bacteremic spread include peritoneal dialysis, pericarditis, bacterial pneumonia, bed sores, prosthetic devices and instrumentation, and skeletal, skin, and soft tissue infections.
5. Common bacteria present in pneumonias that produce a concurrent bacteremia include *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, and *Enterobacter/Klebsiella* spp.
6. Sepsis, septic shock, renal failure, and eventually death are major consequences of septicemia.
7. Antimicrobials, antisepsis therapy, physiologic support (monitor intravascular volume and blood pressure), and anticoagulation agents have been used as treatment for sepsis.
8. Those at risk for polymicrobial bacteremia include immunocompromised patients, especially those with alcoholism, granulocytopenia, extensive burns, diabetes mellitus, and renal failure. Patients with vascular insufficiency resulting from ischemia are also at risk.
9. It is recommended that 10 mL of blood should be drawn from adult patients and placed in 90 mL of liquid medium (1:10 dilution) in each blood culture bottle of a set. This ratio helps reduce the bactericidal effect of serum. Sodium polyanetholsulfonate (SPS) in the culture medium serves as an anticomplement and anticoagulant.
10. The number of samples and time of collection could be highly dependent on the type of bacteremia suspected. In general, collecting three sets of blood cultures within a 24-hour period at 1-hour intervals is appropriate, especially in suspected cases of subacute endocarditis.

Chapter 37

1. a
2. d
3. c
4. c
5. b
6. c
7. d
8. c
9. d
10. b

11. Urine would be collected, likely by clean catch, midstream and plated onto sheep blood agar and a medium selective for gram-negative bacilli, such as MacConkey agar. Plates would be placed at 35° C for at least 16 hours and observed for growth of a predominant organism. After the organism is isolated, it would be identified and antimicrobial susceptibility testing performed.
12. The patient showed signs of a UTI and sepsis. The organism was identified as *Staphylococcus aureus* and was likely spread via the hematogenous route—through the bloodstream. The most common route of infection is through the ascending route, where organisms are introduced via the patient's fecal or skin biota into the urethra.
13. The patient's deteriorating symptoms (delirium, hypotension, fever) in addition to laboratory findings of leukocytosis and positive urine and blood cultures signify urosepsis. The patient should be treated aggressively with empiric antimicrobials until susceptibility results of the blood and urine cultures are completed.
14. A single-episode UTI occurs only once and resolves after antimicrobial therapy, whereas recurrent UTIs occur repeatedly. Recurrent UTIs may involve the same organism (relapse) or a different organism (reinfection).
15. A rapid enzymatic assay, referred to as a *urine dipstick*, is used for initial screening. If reactions indicating the presence of red blood cells, white blood cells (by leukocyte esterase), and/or nitrates are present, the urine then undergoes a microscopic analysis. If the microscopic analysis confirms the presence of white blood cells (pyuria) and bacteria (bacteriuria), or yeasts, cultures are performed. A diagnostic algorithm is useful so only urine samples with a high probability of microbial growth are cultured, which saves cost and laboratory time and gives the clinician insight in determining whether or not the patient has a true UTI to help guide the treatment plan.
16. The minimum incubation time is 16 hours, but the optimal time is 24 hours at 35° C.
17. With longer incubation times, overgrowth of normal biota can obscure a true pathogen. This can result in time lost as additional days are needed to separate normal microbiota from pathogens, and the clinical relevance of the urine culture becomes more confusing.
18. Specimens with multiple species (three or more) likely represent contamination.
19. Routine urine cultures do not include the recovery of fastidious organisms, such as *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, or *Ureaplasma urealyticum*. Symptoms produced by these organisms are difficult to differentiate from those of a true UTI.
20. Sexually active females have a higher rate of *Staphylococcus saprophyticus* infection than other populations. Anyone who is sexually active is also at risk for an STI causing UTI symptoms. A middle-aged man would most likely have common gram-negative pathogens, such as *Escherichia coli*, *Klebsiella* spp., and *Enterobacter* spp. An elderly woman could have yeast present, *Gardnerella vaginalis*, and, if catheterized, possibly *Pseudomonas* spp. and *Proteus* spp. A renal transplant patient can have BK, JC, or adenoviral infections that can present like bacterial infections.
21. The presence of dysuria suggests a UTI while fever, chills, nausea, and vomiting indicate possible sepsis. The UTI could be attributed to hematogenous spread of the infectious agent. The patient's history of a recent organ transplant and likely immunosuppressive therapy places this patient at risk for sepsis. A urinalysis and urine and blood cultures should be performed.

Chapter 38

1. Pelvic inflammatory disease (PID) relates to several different syndromes affecting the uterus, fallopian tubes, and other reproductive organs. PID presents with several vague symptoms, ranging from mild to severe, therefore infection may not be recognized until late in the disease process. The most common complaint is lower abdominal pain, but other potential signs and symptoms include fever, unusual vaginal discharge, painful intercourse, painful urination, or irregular menstrual bleeding. PID can damage the fallopian tubes and tissues in and near the uterus and ovaries while leading to serious consequences, including infertility, ectopic pregnancy, abscess formation, and chronic pelvic pain.
2. There are two common causes of vulvovaginitis: yeast infection (candidiasis) and parasitic infection (trichomoniasis). Women with candidiasis typically present with pruritus (itchiness), although they may also describe painful intercourse and soreness. They have a moderate amount of clumped, adherent discharge (cottage cheese consistency) with no odor. Microscopy performed on a discharge specimen will show budding yeast or fungal elements on the saline wet preparation and the 10% KOH preparation. Women with trichomoniasis may describe soreness and painful intercourse. The discharge is profuse, with a greenish-yellow color and frothy appearance. Application of KOH to this discharge will also produce a fishy smell. Microscopic examination of the discharge can confirm the disease by viewing motile trichomonads in the saline wet mount, but nothing significant is visible in the KOH preparation. Bacterial vaginosis (BV), a polymicrobial infection associated primarily with anaerobic bacteria, is not a true inflammation. Women with BV may have some slight abdominal pain, with a moderate white or gray discharge. By applying KOH to the discharge, a distinctive odor (fishy smell) is noticeable because of the increased vaginal pH. On microscopy, the saline wet preparation will demonstrate clue cells, whereas nothing significant is visible with the KOH preparation. Women with trichomoniasis may describe soreness and painful intercourse. The discharge is profuse, with a greenish-yellow color and frothy appearance. Application of KOH to this discharge will also produce a fishy smell. Microscopic examination of the discharge can confirm the disease by viewing motile trichomonads in the saline wet mount, but nothing significant is visible in the KOH preparation.
3. Tracing of sexual contacts notifies those individuals who may be at risk, with the intent of screening them for STIs as well. In this manner, potentially infected

individuals who are asymptomatic can receive treatment much earlier and reduce the overall complications of a delayed or untreated infection. In addition, treatment of asymptomatic carriers might reduce the spread of the infection.

4. Because STIs are transmitted in the same manner, individuals diagnosed with a specific STI are at risk of infection with another STI. Testing for other STIs should be based on specific risk factors of the individual. However, at a minimum, all diagnosed patients should also be tested for HIV infection.
5. Because of the serious emotional consequences of informing a patient that they have an STI, it is imperative to ensure that laboratory results are confirmed. The most common STIs requiring a confirmatory test are syphilis and HIV infection. Syphilis testing uses a nontreponemal (nonspecific) antibody test such as the rapid plasma reagin test because of its high sensitivity, followed by a treponemal antibody test such as the *Treponema pallidum* particle agglutination test, which is very specific. Infection with HIV is initially screened by an HIV-specific ELISA assay for HIV-1/HIV-2 antibody and p24 antigen. An initial HIV ELISA reactive result should be repeated by an HIV-1/HIV-2 antibody differentiation immunoassay.
6. A variety of preventive measures can help to reduce the spread of STIs. Educating people at greatest risk of developing infection helps them to make better decisions concerning their sexual activities and social interaction. Also, once diagnosed, community and public health officials are notified to help identify and locate all sexual contacts of the individual to get them tested as well. Public health officials also promote safe sexual practices by recommendations such as using protective barriers, prophylaxis, and changing lifestyle habits, such as serosorting, in which HIV-positive individuals only have sexual relations with other HIV-positive individuals.
7. There are two main differences of clinical presentation. Chancres are typically painless, so the individual may not even be aware that he or she is infected. Such lesions are firm and singular in nature, with a sharp demarcated border and a red smooth base. Chancroids are often soft, painful lesions that may appear singly or in groups of three, with an erythematous border and a yellow gray base.
8. Molecular testing has vastly increased the sensitivity and specificity of detection in comparison with culture and a much faster turnaround time for results. Different targets of amplification have been developed to identify other disease agents that may have been difficult to detect before.
9. The symptoms of urethritis are similar, whether caused by *Neisseria gonorrhoeae* or *Chlamydia trachomatis*. In either case, there is urethral inflammation, dysuria, and a discharge. Infected males are typically symptomatic, whereas many females remain asymptomatic. The main discriminating factor is the appearance of the discharge. Gonococcal infections generally yield a purulent discharge; nongonococcal infections may demonstrate a cloudy or watery discharge. The discharge from symptomatic males can be Gram stained. Specimens that show many white blood cells and intracellular gram-negative diplococci provide strong evidence of gonococcal diagnosis because no diplococci are present in the nongonococcal form. However, there are saprophytic *Neisseria* spp. in the female genital tract that might give a similar Gram stain result.
10. Over 150 genotypes of HPV are known, but only 9 of them are included in the vaccine. There are two main groups of genotypes: low risk (e.g., types 6 and 11) and 14 high risk genotypes (e.g., types 16, 18, 31, 33, and 45) for developing cancer. This vaccine protects against nine HPV types: 6, 11, 16, 18, 31, 33, 45, 52, and 58. Since 2016, this has been the only HPV vaccine available in the United States.
11. Patients with HIV infection may also be infected with another STI and should be screened appropriately, depending on their individual risk factors. Screening for tuberculosis is also important because a dual infection speeds up the development of AIDS. Newly confirmed infected patients should have HIV viral load and CD4⁺ count tests performed to establish a baseline of the patient's status, which can be compared with future results to determine the efficacy of treatment.
12. Both of these viruses are considered bloodborne pathogens, but they can be spread by many different mechanisms. Hepatitis B virus is spread via contact with blood, semen, vaginal secretions, and other body fluids of an infected individual. Sexual transmission is responsible for nearly 50% of all HBV infections in the United States. Hepatitis C virus is spread through contact with an infected person's blood and through sexual transmission. Therefore when patients are confirmed to have an STI, they may also require screening for HBV and HCV, depending on their risk factors.
13. All of the herpesviruses have the capability to establish a latent infection. Following productive infection by HSV at the site of inoculation, the virus spreads to and enters sensory neurons, forming a reservoir of virus for recurrent infection, disease, and transmission to other individuals. Reactivation of the virus from the latent state is caused by several triggers, most of which are stress-related. As the virus travels along the nerve axon, lesions occur at the dermatome, which is the area of skin innervated by a single posterior spinal nerve. Thus recurrence of infection occurs at the same site as the initial infection.
14. The declining rate of syphilis was most likely related to the epidemic of HIV infections that encouraged individuals to practice safe sex. Now that many of the younger generation see famous infected individuals who appear healthy, they feel as though HIV can be cured in their lifetime. This attitude has led to increasingly high-risk sexual behaviors.
15. The increasing level of resistance may lead to greater spread of the disease and a reduction in the number of alternatives for treatment. Molecular testing has replaced culture techniques because of increased sensitivity and specificity, ease of testing, multiple specimen types, and rapid turnaround time. Once an individual was diagnosed, the patient would usually respond to antimicrobial therapy. Now, with increasing drug resistance, the only way to detect such isolates

would be to recognize that a patient has failed therapy, and therefore actual culture and susceptibility testing will be required. This extends the period of testing before identifying the appropriate therapy and potential for long-term complications, especially if a patient does not return for follow-up care.

Chapter 39

1. The splenectomy that the patient underwent following the traffic accident placed her at an increased risk for infection, particularly from encapsulated organisms such as *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Neisseria meningitidis*.
2. Malignancy can cause deficiencies in both humoral and cellular immunity. Tumor cells can destroy the normal function of certain cell types in the body, or they may produce cytokines that cause immunosuppression. Cytotoxic drugs given to patients with malignancy can break down mucosal barriers and impair immune response.
3. Although a decrease in the number of neutrophils is the most common hematologic disturbance, inadequacy of neutrophil function, such as the inability to migrate to sites of inflammation, is another cause of increased infections among such patients. The inability of neutrophils to phagocytize or kill ingested organisms also predisposes patients to infection and places them at risk of infection from endogenous organisms.
4. Many organisms are of concern in women who are pregnant. *Escherichia coli* and other gram-negative bacilli can cause urinary tract infections, and *Candida* spp. can cause vaginal yeast infections. Several organisms can cause congenital infections, including *Toxoplasma gondii*, rubella virus, cytomegalovirus, herpes simplex virus, varicella zoster virus, HIV, parvovirus B19, hepatitis B virus, *Treponema pallidum*, *Streptococcus agalactiae*, and *Listeria monocytogenes*. Several organisms can be transmitted during labor and delivery and may adversely affect the baby, including *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *S. agalactiae*, *Escherichia coli*, human papillomavirus, cytomegalovirus, and herpes simplex virus.
5. Several changes in immune function occur as the body ages, increasing its susceptibility to infections and malignancy. The qualitative decline of cellular immune defenses (function) predisposes older individuals to various types of infections, including respiratory, gastrointestinal, urinary tract, and soft tissue infections. Because of decreased tumor surveillance by immune and nonimmune mechanisms, the occurrence of malignancy in this population is also increased.
6. Organisms associated with causing infection in patients with cystic fibrosis include *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Haemophilus influenzae*, *Burkholderia cepacia*, and *Aspergillus fumigatus*. Organisms associated with causing infection in patients with ventilator-associated pneumonia include *Streptococcus pneumoniae* and other streptococci, *H. influenzae*, *S. aureus*, *P. aeruginosa*, *Klebsiella*, *Enterobacter*, *Serratia*, *Acinetobacter*, *Stenotrophomonas*, and *B. cepacia*.

7. Chemotherapy and radiation therapy adversely affect the immune response by damaging white blood cells. These cells are involved in phagocytosis, humoral-mediated immunity, and cell-mediated immunity.
8. b
9. d
10. a

Chapter 40

1. Lyme borreliosis, or Lyme disease, is associated with erythema migrans. *Borrelia burgdorferi* is the causative agent of erythema migrans.
2. Relapsing arthritis of large joints, including the knees, shoulders, and elbows, is the most common clinical manifestation of late, persistent Lyme borreliosis.
3. Cats and dogs are the two animals most often associated with pasteurellosis. *Pasteurella multocida* can be found as normal biota in the oral cavity of these animals.
4. Patients who have been splenectomized or are suffering from cancer are at increased risk. Intravenous drug users may be at increased risk as well.
5. *Bartonella henselae* is the primary causative agent of cat scratch disease. A small number of cases may also be associated with *Bartonella clarridgeiae* and *Bartonella quintana*.
6. Protective antigen (PA) and edema factor (EF) are responsible for the life-threatening edema in pulmonary anthrax. PA must bind with EF to make it biologically active and serves as a transport protein to carry EF into the host cell. EF is an adenylate cyclase that causes edema.
7. The four species are *Brucella melitensis* (goats), *Brucella abortus* (cattle), *Brucella canis* (canines), and *Brucella suis* (swine).
8. Anicteric leptospirosis is the milder, less lethal form of the disease, which is typically biphasic. The characteristic symptom for this disease is pink eyes. Icteric is much more likely to be life threatening, with a mortality rate of 15% to 40%. Although this form may initially present similarly to the anicteric form, on about the third day the patient begins showing signs of hemolysis, jaundice, and renal failure as the leptospire multiplies in the liver and kidney.
9. *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis* are the causative agents of HGA and HME, respectively. The severity of these infections is increased in patients with impaired splenic function or those with a history of splenectomy.
10. The typhus group includes *Rickettsia typhi* and *R. prowazekii*.
11. Rocky Mountain spotted fever is the most severe of the rickettsial infections. By infecting endothelial cells, the organism causes vascular damage to the internal organs, including the brain, heart, lungs, and kidneys.
12. Emerging zoonotic diseases can jump from animals to humans. They can be newly discovered agents, known disease agents that have moved to a new geographic location, or known diseases that have undergone a mutation, making them more pathogenic (e.g., increased drug resistance). The cost associated with zoonotic

outbreaks in the past decade has been estimated at \$200 billion. Massive economic loss has also been associated with the COVID-19 pandemic that started in 2019 and is still ongoing.

13. c
14. d
15. a
16. b
17. d
18. d
19. b
20. c
21. a
22. c

Chapter 41

1. b
2. c
3. d
4. a
5. d
6. Specimens from the conjunctiva are the most commonly submitted specimens from ocular sites. The most frequent isolates are *Staphylococcus aureus*, coagulase-negative staphylococci, *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Pseudomonas aeruginosa*.
7. Gram, Giemsa, calcofluor white, acid-fast, and immunofluorescent stains are commonly used on ocular material.
8. The topical route is the most common route of administering antimicrobial agents in the treatment of eye infections. In more serious infections, systemic treatment may be required.
9. *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, and herpes simplex viruses are sexually transmitted agents that can also affect the eyes.
10. Corneal scraping material collected by an ophthalmologist should be plated directly to culture media and used to make a smear. Plates and slides should be transported immediately to the laboratory.
11. Soft lenses contain a large percentage of water, allowing pathogens to survive and multiply. Improper handling of lenses (i.e., poor hygiene) and failure to follow the manufacturer's recommended procedures on how to keep lenses, lens solutions, and cases from being contaminated place the lens wearer at risk for eye infection. Proper handwashing and good hygiene practices to prevent lens contamination may reduce the risk. Avoid wearing contact lenses in water.
12. a
13. b
14. c
15. c

Selected bacteriologic culture media

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A wide variety of basal, enrichment, selective, and differential media are available to the clinical microbiology laboratory. Each of these media is intended to aid the laboratory scientist in the isolation, cultivation, and identification of clinically significant organisms from patient specimens. Efficient laboratory performance depends not on maintaining a vast array of media for routine use, but on the ability to make wise choices when selecting a routine media menu—choices that should be dictated by the factors discussed in [Chapter 6](#). This appendix provides information about a select number of bacteriologic media cited in the bacteriology sections of this text. Most media detailed in this appendix are commercially available as a dehydrated powder or in ready-to-use form. Sterility and performance tests (quality controls) should be performed, as discussed in [Chapter 5](#).

Alkaline peptone water

Alkaline peptone water is an enrichment medium useful in the recovery of *Vibrio* and *Aeromonas* spp. from stool specimens. The alkaline pH of this medium allows uninhibited replication of these species while temporarily suppressing the replication of many commensal intestinal bacteria. The sodium chloride (NaCl) concentration is 1%.

Some formulations recommend adjusting to pH 9.0 and adding NaCl to a concentration of 3% (alkaline peptone salt broth) to specifically recover the vibrios. Alkaline peptone water cultures should be incubated at 35°C and subcultured to thiosulfate citrate bile salt sucrose (TCBS) agar within 12 to 18 hours.

American Trudeau Society medium

American Trudeau Society (ATS) medium is an egg-based medium used for the isolation of mycobacteria, particularly *Mycobacterium tuberculosis*, from clinical specimens. The eggs provide fatty acids, and potatoes are included to provide

a carbon source. Malachite green is incorporated into the medium, which is slightly inhibitory for bacteria that are part of the normal microbiota.

Amies transport with and without charcoal

Amies transport medium, a modification of the Stuart transport medium, is commonly used to maintain the integrity of the specimen from the time of collection until laboratory processing of the sample. This medium usually contains substances that do not promote multiplication of microorganisms but ensure their preservation. Swab collection systems may contain this medium. Amies transport medium may contain charcoal that absorbs fatty acids given off by the swab or present in clinical specimens that can be detrimental to the survival of *Neisseria gonorrhoeae* and *Bordetella pertussis*. The medium contains 0.3% NaCl for optimal preservation of *N. gonorrhoeae*. Potassium, calcium, and magnesium salts control permeability of bacterial cells by maintaining osmotic equilibrium while phosphate buffer maintains the pH of the medium.

Bacteroides bile esculin agar

Bacteroides bile esculin (BBE) agar is a selective differential agar used for the isolation and identification of members of the *Bacteroides fragilis* group. The incorporation of oxgall (bile salts) separates bile-resistant species (growth) from bile-sensitive ones (no growth), whereas the 1% esculin, in conjunction with ferric ammonium citrate, differentiates between the isolates that grow based on the isolate's ability (positive) or inability (negative) to hydrolyze esculin. If the organism produces esculinase, it will hydrolyze esculin into esculetin and glucose and metabolize the glucose. Esculetin reacts with the ferric ammonium citrate to form an insoluble iron salt in the medium surrounding the positive colonies, causing it to turn

dark brown or black. Plated medium should be inoculated, streaked for isolation, and incubated anaerobically at 35°C.

Bile esculin agar

Bile esculin agar is a selective differential agar used to isolate and identify group D streptococci and the enterococci. Oxgall (bile salt) is the selective ingredient that inhibits the growth of most gram-positive organisms, whereas esculin is the differential component. All group D streptococci and enterococci can grow in the presence of bile and hydrolyze esculin. Products of esculin hydrolysis (esculetin) react with ferric citrate in the medium to produce insoluble iron salts. Deposition of the iron salts results in a blackening of the medium. Test results must be interpreted in conjunction with Gram stain morphology because *Listeria monocytogenes* and a small number of other organisms also produce positive reactions.

The addition of vancomycin to the traditional bile esculin medium can be used to detect vancomycin-resistant streptococci and enterococci. Azide is added to some formulations to inhibit gram-negative organisms. When azide is added, the concentration of bile is decreased to make the medium less inhibitory to non-group D streptococci. Bile esculin medium should be inoculated, incubated aerobically at 35°C, and observed for growth. Darkening of the medium indicates esculin hydrolysis.

Bismuth sulfite agar

Bismuth sulfite agar is a selective medium for the isolation of *Salmonella* spp. The selective ingredients are bismuth sulfite and brilliant green, which inhibit the growth of gram-positive bacteria, most lactose-fermenting intestinal normal microbiota (coliforms), and *Shigella* spp. (other than *S. flexneri* and *S. sonnei*). Although this is not a differential medium in the strictest sense, the ferrous sulfate in this medium is reactive with hydrogen sulfide (H₂S) to produce ferric sulfide, which is deposited in the bacterial colony as a black, insoluble precipitate. Typically, *Salmonella* serotype Typhi colonies are black and surrounded by a metallic sheen, whereas *Salmonella* serotype Gallinarum, *Salmonella* serotype Choleraesuis, and *Salmonella* serotype Paratyphi colonies are light green on this medium. When bismuth sulfite agar is used to isolate *Salmonella* from feces and other clinical specimens, the parallel use of a less inhibitory medium is recommended, because bismuth sulfite agar can inhibit or partially inhibit the growth of some *Salmonella* strains.

This medium cannot be autoclaved and must be used on the day it is prepared. Plated medium should be inoculated with fecal or enrichment broth materials, streaked for isolation, and incubated at 35°C for 48 hours.

Blood agar, anaerobic, CDC

The Centers for Disease Control and Prevention (CDC) anaerobic blood agar (CDC, Atlanta, GA) is an enriched medium useful for the isolation of fastidious anaerobes. It is better for the isolation of anaerobic gram-positive cocci than for other

anaerobes. It has a tryptic soy agar base and contains yeast extract, L-cysteine, hemin (factor X), 5% sheep blood, and vitamin K₁. This medium can be stored for up to 6 weeks if sealed in a cellophane bag and stored at 4°C. Plates should be inoculated, streaked for isolation, and incubated anaerobically at 35°C for 48 hours.

Blood agar, anaerobic, with kanamycin and vancomycin

Kanamycin and vancomycin (KV) blood agar is a variation of CDC anaerobic blood agar made semi-selective by the addition of the antimicrobials kanamycin and vancomycin. It is useful in the primary isolation of obligate gram-negative anaerobes, particularly *Bacteroides* spp., from specimens with a mixed bacterial population.

KV blood agar plates sealed in bags can be stored at 4°C for up to 4 weeks. Plates should be inoculated, streaked for isolation, and incubated anaerobically at 35°C for 48 hours. Inoculated plates that lack growth at 48 hours may be re-inoculated, depending on the situation.

Blood agar, rabbit

Rabbit blood agar is an enrichment medium particularly useful in the recovery and the demonstration of β-hemolysis by *Haemophilus* spp. and *Gardnerella vaginalis*.

Blood agar, sheep

Sheep blood agar (SBA) is a routine medium used to cultivate a wide variety of moderately fastidious bacteria. An infusion agar, tryptic soy agar base, or Columbia agar base is enriched by the addition of 5% to 10% defibrinated sheep, rabbit, or human blood. Sheep blood, however, has proven to be the most versatile enrichment additive. Incorporation of the blood not only provides enrichment for the growth of bacteria but it also allows for differentiation based on the detection and characterization of hemolytic activity. Plates should be inoculated, streaked, and incubated as dictated by the specific application.

Bordet-Gengou blood agar

Bordet-Gengou (B-G) blood agar is a selective enrichment medium for the isolation of *Bordetella pertussis* and *Bordetella parapertussis* from clinical specimens. Peptone is used in the base medium and is enriched by the addition of glycerol; potato infusion; and sterile, defibrinated sheep blood. Selectivity of the medium is achieved by adding penicillin, methicillin, or cephalixin to the medium.

The antibiotics should be added aseptically just before the addition of the blood enrichment. Blood enrichment of between 15% and 30% is appropriate. The source of the sterile defibrinated blood obtained is not critical, but properly prepared plates should be cherry red, moist, and bubble-free. Complete plated medium should be used immediately if

possible. If this is not possible, plates can be maintained for about 1 week at 4°C if they are tightly sealed.

The medium can be inoculated by rolling the nasopharyngeal swab specimen over one third of the plate surface and then streaking for isolation with a loop. The specimen should be inoculated in this fashion onto B-G plates with and without antimicrobial agents. Inoculated plates should be incubated at 35°C with 5% to 10% carbon dioxide (CO₂) and increased humidity and then examined at 48 hours. Plates that have no growth at 48 hours should be reincubated. Plates must be held for 5 days, but not more than 7 days, before they can be regarded as negative for the organism.

Brain-heart infusion broth

Brain-heart infusion (BHI) broth is an enriched medium suitable for the cultivation of nonfastidious and moderately fastidious microorganisms. This broth medium is recommended for the cultivation of pneumococci for the bile solubility test. Brains and beef heart provide nutrients, and peptones, glucose, NaCl, and buffers also are added. This formulation is used as a blood culture medium. NaCl (6.5%) can be added to differentiate the salt-tolerant enterococci from the streptococci that are inhibited by the high salt concentration. This broth also can be used to prepare inocula for antimicrobial susceptibility testing and detecting vancomycin-resistant enterococci.

Brucella laked sheep blood agar, anaerobic, with kanamycin, vancomycin, and vitamin K

Anaerobic, laked sheep blood agar with kanamycin, vancomycin, and vitamin K (KVKL) is a selective enrichment medium recommended for the isolation of *Bacteroides* and *Prevotella* species from clinical specimens. This medium is particularly helpful in the isolation of *Prevotella melaninogenica* because pigment production is enhanced. Laked erythrocytes (lysed by freezing) and vitamin K constitute the enrichment ingredients; the antibiotics inhibit all cocci and facultative gram-negative bacilli, except the pseudomonads. Other microorganisms can also grow such as some yeast; therefore a Gram stain should always be performed to help differentiate isolates. Plated medium should be inoculated, streaked for isolation, and incubated anaerobically at 35°C for a minimum of 48 hours.

Buffered charcoal yeast extract agar

Buffered charcoal yeast extract (BCYE) agar is an enrichment medium useful in the isolation of *Legionella* spp. from clinical specimens. Ferric pyrophosphate provides iron that is required by the organism. Yeast extract, α -ketoglutarate, and L-cysteine enhance the growth of *Legionella* and other organisms. Activated charcoal is incorporated to absorb toxic compounds that accumulate because of the organism's metabolism or are present following preparation of this

medium. This medium can also be used to isolate *Francisella* and *Nocardia* spp.

Plated media can be stored in plastic bags, away from light, at 4°C for up to 4 weeks. Plated media should be inoculated, streaked for isolation, and incubated at 35°C in a CO₂ incubator. Cultures should be checked daily for up to 2 weeks, and incubator humidity should be monitored to prevent excessive drying of plates. *Legionella* colonies may not be grossly visible until 3 to 5 days after inoculation.

Several modifications of BCYE exist. In one formulation, L-cysteine is omitted. Because *Legionella* spp. require L-cysteine, they will not grow on this medium. Comparison of growth on this L-cysteine-deficient medium with that on regular BCYE can help determine whether an isolate is *Legionella* spp. or another type of gram-negative rod. The addition of antimicrobial agents to BCYE makes the medium selective. Commonly used antimicrobials include cefamandole or vancomycin to inhibit gram-positive organisms; colistin or polymyxin B to inhibit gram-negative bacilli, especially pseudomonads; and anisomycin for fungal inhibition. The Wadowsky-Yee modification uses glycine and polymyxin B to inhibit gram-negative organisms, vancomycin to inhibit gram-positive cocci, and anisomycin to inhibit fungi. This medium is useful in recovering *Legionella* spp. from body sites that contain mixed microbiota. The Wadowsky-Yee modified BCYE also has bromocresol purple and bromothymol blue, which help differentiate among colonies. *L. pneumophila* colonies are light blue, with a pale green tint.

Burkholderia cepacia agar

Burkholderia cepacia agar was developed to isolate *B. cepacia* selectively from respiratory specimens collected from patients with cystic fibrosis. The medium was originally named *Pseudomonas cepacia* agar, which corresponds to the former name of the bacterium. *B. cepacia* grows more slowly than other bacteria and has variable colony morphologies on routine media. As a result, other organisms often overgrow *B. cepacia*, and isolation and detection of *B. cepacia* were problematic. Crystal violet, bile salts, polymyxin B, and ticarcillin are incorporated to inhibit most gram-positive and gram-negative organisms. The medium contains inorganic salts, peptones, pyruvate, and phenol red. *B. cepacia* can grow on this medium and use pyruvate as a carbon source. The breakdown of pyruvate results in the production of alkaline byproducts. The increased pH changes the phenol red indicator from dull yellow to hot pink.

Burkholderia cepacia selective agar

Burkholderia cepacia selective agar (BCSA) is used to select for the isolation of *B. cepacia* in respiratory specimens taken from patients with cystic fibrosis. The base consists of trypticase peptone, yeast extract, sucrose, lactose, and NaCl. Polymyxin B, gentamicin, vancomycin, and crystal violet are the selective agents. *B. cepacia* has variable colony morphology on this medium, from smooth to rough, dry or moist, or purple because of the absorption of crystal violet. In addition, colonies may have a surrounding yellow zone because of carbohydrate fermentation or a pink zone because of peptone

breakdown. BCSA is better for selectively isolating *B. cepacia* than *B. cepacia* agar or oxidative-fermentative base, polymyxin B, bacitracin, lactose (OFBBL) media. This medium can also be used for the isolation of rapidly growing mycobacteria. Inoculated BCSA plates should be incubated aerobically at 35°C and checked every 24 hours for growth. Plates should be incubated for at least 72 hours before they are discarded if there is no growth.

Cary-Blair transport medium

Cary-Blair transport medium is a semi-solid medium designed to preserve and transport clinical samples especially stool and rectal swabs. Its purpose is to enhance the survival and recovery of bacterial enteric pathogens. The low nutrient content in the medium and sodium thioglycolate to inhibit oxidation prevent bacterial multiplication while still maintaining viability and prevent colon biota overgrowth of enteric pathogens. Disodium phosphate is included as a buffering agent, and NaCl is added to maintain proper osmotic pressure. The alkaline pH reduces the damage to bacterial cells when acid is produced.

Campylobacter blood agar

Campylobacter blood agar (Campy-BA) is a selective enrichment medium useful in the isolation and cultivation of *Campylobacter* spp. from stool specimens. *Brucella* agar serves as the base medium for Campy-BA because it contains sodium bisulfite, which lowers the redox potential, thereby enhancing the recovery of microaerophilic organisms such as *Campylobacter* spp. Ten percent sheep blood enriches the basal medium, and an antimicrobial mixture makes the medium selective. Although minor variations exist in the composition of the antimicrobial mixture, most formulations have incorporated vancomycin to inhibit gram-positive cocci, trimethoprim to inhibit swarming strains of *Proteus*, polymyxin B to inhibit gram-negative bacilli, and amphotericin B to inhibit filamentous fungi and yeasts. Cefoperazone is being promoted to replace cephalothin. Cefoperazone has antipseudomonal activity (lacking in cephalothin) and is more effective against members of the order Enterobacterales. After inoculation with stool, the medium should be incubated in a microaerophilic and capnophilic environment at 42°C. *Campylobacter* species generally grow as flat, gray, nonhemolytic colonies, or they may be raised and mucoid. Some isolates may be tan or slightly pink. The colonies may appear swarming or spreading across the surface of the plate.

Campylobacter charcoal differential agar

Campylobacter charcoal differential agar is less inhibitory for *Campylobacter* spp. than other selective media used to isolate *Campylobacter* and is also more inhibitory for organisms found as normal fecal microbiota. Preston agar is the base that is supplemented with beef extract and peptones. Cefoperazone and sodium deoxycholate are added as inhibitors. Blood is

not added to this formulation. Once inoculated with stool, this plate should be incubated in a microaerophilic and capnophilic environment at 42°C.

Campylobacter thioglycolate broth

Campylobacter thioglycolate broth (Campy-Thio) is a selective liquid medium used to enhance the isolation of *Campylobacter* spp. It also is used as a holding or transport medium for these organisms. The base medium is thioglycolate broth with 0.16% agar, and the selective component is the antimicrobial formulation used in Campy-BA.

Cefsulodin-Irgasan-novobiocin

Cefsulodin-irgasan-novobiocin (CIN), also known as *Yersinia*-selective agar, is used to select for the isolation of *Yersinia enterocolitica* in stool samples. Peptones, beef, and yeast extracts are added as nutrient sources. Sodium deoxycholate, cefsulodin, novobiocin, irgasan, and crystal violet are used to inhibit the growth of other organisms found in stool. Mannitol is the differentiating agent, and neutral red is the pH indicator. *Yersinia* grows on this medium and ferments mannitol, forming clear colonies with a red center described as a "bull's eye." *Aeromonas* spp. also will grow and ferment mannitol, but the *Aeromonas* colonies have a pink center and an uneven clear periphery. Most other organisms will not grow on this medium.

Cetrimide agar

Cetrimide agar, also known as pseudosel agar or *Pseudomonas*-selective medium, is used to select for *Pseudomonas aeruginosa* in specimens with mixed microbiota. Also, because it inhibits other *Pseudomonas* spp. (except *P. fluorescens*) and closely related organisms, this test medium can be useful in the differentiation of nonglucose-fermenting, gram-negative rods.

This medium contains cetrimide, also called *cetyl trimethyl ammonium bromide* or *hexadecyltrimethyl ammonium bromide*. Produced from bromine, cetrimide is highly inhibitory and has been used as an antiseptic. If the organism can tolerate cetrimide, it will grow on the medium. Magnesium chloride and potassium sulfate stimulate the production of pyocyanin, the blue pigment, and pyoverdine, the yellow-green pigment, characteristically produced by *P. aeruginosa*. Under ultraviolet light, colonies of *P. aeruginosa* will fluoresce a yellow-green because of pyoverdine production stimulated by the low iron content of the medium.

Chocolate agar

Chocolate (CHOC) agar is an enrichment agar that is especially useful in promoting the growth of *Haemophilus* and other fastidious bacterial species. This medium can be made by adding sheep blood while the basal medium is warm enough to lyse the red blood cells, releasing hemoglobin and nicotinamide adenine dinucleotide (NAD). Alternatively, sheep blood may be replaced by 2% hemoglobin and a

chemical supplement solution, such as BBL IsoVitaleX enrichment (Thermo Fisher Scientific, Waltham, MA). The complete medium must contain cell-free hemoglobin and NAD. Either enrichment results in a chocolate brown-colored medium. Plated medium can be stored at 4°C. Plates should be inoculated, streaked for isolation, and incubated at 35°C in CO₂.

CHROMagars

CHROMagars (DRG International, Springfield, NJ) are selective, differential, chromogenic media that have been developed for the isolation and identification of yeast, *Staphylococcus aureus*, *Escherichia coli* O157:H7, and other organisms. Peptone and glucose are present in the basal nutrient agar, to which are added proprietary chromogenic mixtures, antimicrobial agents, and other additives. As a result, particular bacteria will have a typical and predictable colored colony that can be distinguishable from other colonies. These media can be used as primary isolation plates, such as the CHROMagar Orientation that is suggested for urine cultures in place of a blood agar plate, MacConkey agar, or both.

Citrate agar, Simmons

Simmons citrate agar is useful in differentiating gram-negative enteric bacilli. Similar in principle to acetate agar, citrate replaces acetate in this medium, and differentiation is based on the isolate's ability or inability to use citrate as its sole source of carbon. Organisms capable of using citrate also use the medium's ammonium salt as a nitrogen source. The breakdown of the ammonium salt results in a shift of the pH into the alkaline range. At alkaline pH, the incorporated pH indicator bromothymol blue changes from green to blue.

This medium should be inoculated by streaking the organisms onto the slant with a sterile loop or needle. A light inoculum should be used because dead organisms from a heavy inoculum can be a carbon source, producing a false-positive reaction. Inoculated slants should be incubated at 35°C for up to 4 days and monitored daily for the expected color change. Organisms that can use citrate (positive) will grow on the slant and produce a blue color in the slant. Organisms that cannot use citrate (negative) will not grow on the slant.

Columbia agar with and without 5% sheep blood

Columbia agar is a basal nutrient agar that contains peptones derived from casein and meat as well as beef and yeast extracts. The basal medium is suitable for cultivation of several aerobic and anaerobic bacteria found in clinical materials. Additionally, it provides an efficient base for preparation of a variety of enrichment agars that support the growth of more fastidious aerobes and anaerobes (e.g., 5% sheep blood). The addition of sheep blood allows for the detection of hemolytic reactions, although sometimes the β -hemolytic streptococci may look α -hemolytic because of the high carbohydrate content of this medium. Furthermore, the sheep blood will

provide X factor for organisms that require it, but NADase present in the blood will destroy the V factor, so organisms requiring V factor will not grow on this medium.

Columbia agar with colistin nalidixic acid

Columbia agar with colistin nalidixic acid (CNA), referred to as Columbia CNA agar, is an enrichment-selective medium suitable for the isolation of gram-positive cocci such as staphylococci, streptococci, and pneumococci from specimens that might also be expected to contain gram-negative bacilli, especially *Proteus* spp. Sheep blood is the usual enrichment ingredient, and colistin and nalidixic acid are incorporated to inhibit gram-negative overgrowth. Sheep blood also allows detection of hemolytic reactions especially important in the presumptive identification of streptococci.

Cooked meat (chopped meat glucose) medium

Cooked meat medium is useful in the cultivation of anaerobes, especially pathogenic *Clostridium* spp. This medium contains solid meat particles and is excellent for initiating growth from a very small inoculum as well as sustaining culture viability over long periods. It is useful for the cultivation of mixed cultures because all organisms are supported, whereas overgrowth by the more rapid growers is retarded. Tubed medium should be stored at room temperature, but stored tubes should be placed in flowing steam or a boiling water bath for 10 minutes to drive off dissolved gases and then cooled rapidly before inoculating. Cooled tubed media should be inoculated and incubated in a manner appropriate for the species being isolated or subcultured. The digestion of the meat particles indicates the proteolytic activity of cultured organisms. Saccharolytic clostridial species typically produce acid with gas.

Cycloserine cefoxitin fructose agar

Cycloserine cefoxitin fructose agar (CCFA) is a selective, differential medium useful in the isolation and identification of *Clostridioides difficile* from stool specimens of patients suspected of having antibiotic-associated diarrhea with pseudomembranous colitis. The selective antimicrobial ingredients, cycloserine and cefoxitin, inhibit the growth of intestinal normal biota by interfering with cell wall synthesis in gram-positive and gram-negative bacteria. Indigenous bacteria are inhibited, but *C. difficile* is not. Although cycloserine and cefoxitin are incorporated for their selective properties, fructose and a pH indicator, neutral red, are included to confirm that the isolates can ferment this sugar.

A variation of this medium is made with mannitol rather than fructose and uses bromothymol blue indicator. A second variation adds egg yolk suspension so lecithinase and lipase activities can be detected. *C. difficile* grows as a yellow colony and turns the surrounding medium yellow. Plates should be

held for 7 days. If the colonies are viewed under an ultraviolet light, *C. difficile* will fluoresce a gold-yellow color.

Cystine-glucose blood agar

Cystine glucose blood agar is a highly enriched medium used for the recovery of *Francisella tularensis*. The agar base contains beef heart infusion; peptone and L-cystine that provide nitrogen; vitamins; and amino acids. Glucose is the carbon source, and NaCl maintains the appropriate osmotic balance. Enrichment with 2% hemoglobin from rabbit blood provides additional growth factors.

Cystine tryptic agar with sugar

Cystine tryptic (tryptophan [trypticase]) agar (CTA) is a semi-solid base medium that contains no meat or plant extracts and is free from fermentable carbohydrates. It may be made differential by the addition of a carbohydrate (CTA sugar). CTA sugars are used for the determination of fermentation reactions by fastidious organisms.

Alternatively, carbohydrates are available in the form of differentiation disks that can be aseptically added to the base tubed medium as needed. Sterile, tubed CTA sugars should be inoculated with a heavy inoculum, stabbing to a depth of approximately 2 mm below the medium surface. The caps should be tightly closed and the tubes incubated at 35°C for 24 hours. With phenol red as the pH indicator, fermentation is indicated by a color change in the medium from red to yellow.

Decarboxylase test medium (Moeller)

Decarboxylase test medium with an incorporated amino acid is a differential medium useful in the identification of fermentative and nonfermentative gram-negative bacteria. The differential ingredient is one of three amino acids: lysine, arginine, or ornithine. Decarboxylation of the amino acids yields alkaline end products, which are detected by a change in the color of an incorporated pH-sensitive dye, bromocresol purple. The tubed basal medium (no amino acid) serves as a control for reading the reactions.

Decarboxylase tubes and a control tube should be inoculated from a 24-hour culture using a loop. Inoculated tubes should be overlaid with 4 to 5 mm of sterile mineral oil to avoid oxidative deamination of available protein, which would be falsely interpreted as a positive reaction. Inoculated, overlaid tubes should be incubated at 35°C for up to 4 days. Tubes should be checked daily. Early in the incubation, fermentative organisms will ferment glucose, turning the control and all decarboxylase tubes yellow. For these organisms, as the pH drops, the hydrogen ion concentration becomes optimal for decarboxylase activity in the decarboxylase tubes; the subsequent conversion of the amino acid to amines raises the pH, reversing the yellow to purple, whereas the control tube remains yellow. Nonfermenters do not produce the initial yellow color change, and use of the amino acid is indicated when the amino acid-containing tube becomes a deeper purple than the control.

Deoxyribonuclease test agar

Deoxyribonuclease (DNase) test agar (with or without indicator dye) is a differential medium used to detect the production of a DNase exoenzyme by aerobic bacterial species. The differential ingredient is incorporated DNA. Methods available for the detection of DNA degradation include hydrochloric acid precipitation of undegraded DNA and a color change of an incorporated metachromatic dye, such as toluidine blue or methyl green.

Plated medium should be inoculated using a 1- to 2-cm streak or a spot inoculum approximately 5 mm in diameter. Inoculated plates should be incubated aerobically at 35°C for 18 to 24 hours. Following incubation, DNase activity is detected in one of the following ways:

1. If a basal medium without a metachromatic dye was inoculated, flood the plate with 1 N HCl and look for a zone of clearing around the bacterial growth. If the incorporated DNA is undegraded, it is precipitated by the 1 N HCl, and the medium becomes opaque. If the incorporated DNA is degraded, the nucleotide fragments dissolve in the 1 N HCl, and the medium remains clear. A clear halo around the colony is a positive result.
2. If the medium includes toluidine blue, the blue, DNA-bound dye is released from nucleotide fragments, producing a color change to rose. The medium remains clear blue in negative reactions.
3. If the medium includes methyl green, the green, DNA-bound dye is released from nucleotide fragments, resulting in a loss of color. The medium remains green in negative reactions.

Egg yolk agar

Egg yolk agar (EYA), also known as modified *McClung Toabe agar*, is a differential medium useful in the detection of lecithinase, lipase, and protease activity. Incorporated egg emulsion provides the lecithin, lipids, and proteins to be degraded by these enzymes. Exoenzyme activity is detected on EYA. Lecithinase activity produces a zone of opacity immediately around the growth streak, lipase activity results in an iridescent sheen on or around the surface of colonies, and protease activity is seen as a clearing of the medium around and just beyond the streaked growth area. An organism can produce one or all of these exoenzymes.

Plated medium is inoculated as a single streak across the plate and incubated anaerobically at 35°C for 24 to 72 hours. If the Nagler test is to be performed, one half of the plate surface should be smeared with a few drops of *Clostridium perfringens* type A antitoxin before inoculation. The inoculation streak should then extend across both halves (no antitoxin-antitoxin) of the plate. Plates should be incubated anaerobically at 35°C for 24 to 48 hours. A positive test result is the inhibition of lecithinase activity on the half of the plate with antitoxin. Plated medium not used immediately can be stored at 4°C if it is sealed in plastic bags.

Eosin-methylene blue agar

Eosin-methylene blue (EMB) agar is a selective differential medium useful in the isolation and identification of

gram-negative enteric bacteria. The dyes eosin Y and methylene blue make the medium selective by inhibiting the growth of gram-positive bacteria while allowing the growth of gram-negative ones. The carbohydrates lactose and sucrose are incorporated to allow differentiation of isolates based on lactose or sucrose fermentation. Fermentation is detected by color changes and precipitation of the incorporated dyes as the pH drops. Sucrose serves as an alternative carbohydrate source for slow lactose fermenters, allowing their timely elimination from consideration as possible pathogens. *E. coli*, a rapid lactose fermenter, typically forms blue-black colonies with a metallic greenish sheen. Other Enterobacterales fermenters, such as *Enterobacter*, form pink colonies. Nonfermenter colonies are translucent and colorless or light purple.

Esculin agar

Esculin agar is a differential medium used to determine the ability of an organism to hydrolyze esculin. The hydrolytic product from esculin, esculetin, reacts with the ferric salt present in this medium to precipitate iron compounds and produce a gray to black discoloration of the medium. The medium should be inoculated, incubated aerobically at 35°C, and observed for growth, with darkening of the medium indicative of esculin hydrolysis.

Fletcher semi-solid medium for *Leptospira*

Fletcher semi-solid medium is an enrichment medium recommended for the recovery of leptospiral species in blood, cerebrospinal fluid, urine specimens, and possibly contaminated water and other materials. The enrichment component of this medium is rabbit serum containing hemoglobin.

The lyophilized rabbit serum with hemoglobin is commercially available as *Leptospira* Enrichment, or sterile, pooled, fresh natural rabbit serum may be used. The medium should be aseptically dispensed into sterile, screw-capped tubes (5mL/tube) and stored at room temperature overnight. The complement in the medium must be inactivated by placing tubes in a 56°C water bath for 1 hour on the day following preparation. Cooled inactivated medium should be inoculated with one or two drops of the fluid specimen. Small inocula introduced into multiple tubes are recommended to optimize pathogen recovery and minimize any interference by antibodies in the body fluid specimens. Inoculated tubes should be incubated with caps loosened at 25° to 30°C for 4 to 5 weeks and examined weekly for growth in the form of turbidity at the top of the medium. A loopful of fluid from any tube showing turbidity should be placed on a slide, a coverslip added, and the specimen examined by dark-field microscopy. Medium can be made selective by incorporating 5-fluorouracil. Ellinghausen-McCullough-Johnson-Harris medium can also be used for recovery of leptospires in clinical specimens.

GC-Lect agar

GC-Lect agar (BD Diagnostic Systems) is a selective medium that provides enhanced growth and recovery of *N. gonorrhoeae*

and inhibits contaminating organisms. GC-Lect agar includes inhibitors that prevent overgrowth of pathogenic *Neisseria* spp. in specimens containing contaminating strains resistant to the inhibitors in modified Thayer-Martin (MTM) agar; for example, vancomycin-resistant *Staphylococcus epidermidis*. The medium contains lincomycin, vancomycin, trimethoprim, colistin, and amphotericin B. To improve recovery of *N. gonorrhoeae* strains that are sensitive to vancomycin, the concentration of this antibiotic in GC-Lect agar is decreased compared to MTM.

Gram-negative broth

Gram-negative (GN) broth is a selective enrichment medium used to enhance the recovery of enteric pathogens *Salmonella* and *Shigella* spp., from fecal specimens. The selective ingredients are deoxycholate and citrate salts, which inhibit the growth of gram-positive bacteria and some gram-negative bacilli, while allowing the growth of *Salmonella* and *Shigella* spp. Enrichment is provided by increasing the concentration of mannitol, which temporarily favors the growth of mannitol-fermenting, gram-negative bacilli (*Salmonella* and *Shigella*) over that of the mannitol nonfermenters (e.g., *Proteus*).

Sterile tubed media should be inoculated with fecal material and incubated, with caps loosened, at 35°C. Incubated GN broth cultures should be subcultured onto selective, differential plated media after 6 to 8 hours and again after 18 to 24 hours.

Haemophilus test medium

Susceptibility testing of *Haemophilus* isolates is performed on the enriched *Haemophilus* test medium. This medium has a clear agar base and includes beef, yeast, and casein extracts as well as hematin and NAD. This formulation can also be made as a liquid and used for broth minimal inhibitory concentration (MIC) determinations of *Haemophilus* spp.

Hektoen enteric agar

Hektoen enteric (HE) agar is a selective differential medium used for direct isolation of enteric pathogens from feces and for indirect isolation from selective enrichment broth, e.g., GN broth. The selective ingredients are bile salts at concentrations that not only inhibit the growth of gram-positive bacteria, but also inhibit the growth of many gram-negative organisms that are part of the normal intestinal microbiota. The differential ingredients include lactose, salicin, and sucrose to determine fermentation patterns, detected by the pH indicator bromothymol blue; and ferric salts (sodium thiosulfate, ferric ammonium citrate) to detect the production of H₂S gas.

This medium should not be autoclaved, and overheating should be avoided. Plated medium should be inoculated, streaked for isolation, and incubated aerobically (not in CO₂) at 35°C for 18 to 24 hours. Most nonpathogens ferment one or both of the sugars, and colonies appear bright orange to salmon pink because of the low pH interaction with incorporated dyes. Nonfermenters, such as *Salmonella* and *Shigella*

spp., typically produce green to blue-green colonies. H₂S gas production is detected as a black precipitate that accumulates within the center of colonies.

Kligler iron agar

Kligler iron agar (KIA) can be used to determine whether a gram-negative rod is a glucose or lactose fermenter or both. The medium also tests for gas production during carbohydrate fermentation and H₂S production, both of which are useful in the differentiation of gram-negative rods belonging to the Enterobacterales.

KIA contains glucose and lactose (fermentable carbohydrates), where lactose is present at 10 times the glucose concentration; phenol red (pH indicator); peptone (carbon-nitrogen source); and sodium thiosulfate plus ferric ammonium sulfate (sulfur source and H₂S indicator, respectively). KIA resembles triple sugar iron (TSI) agar, except that it lacks sucrose. By convention, when reading the reactions, shorthand is used—the reaction of the slant is put over the reaction in the butt, and K is used for alkaline and A for acid. The following three carbohydrate fermentation patterns are possible:

1. Alkaline (red) slant and acid (yellow) butt (K/A) indicate that the organism ferments glucose but not lactose. This organism ferments glucose by the Embden-Meyerhof pathway to produce organic acids, changing the pH indicator from red to yellow. Once the glucose has been consumed, the organism then breaks down peptones, producing ammonia. The breakdown of peptones happens only aerobically and results in an increase in pH; thus the slant reverts to red.
2. Acid (yellow) slant and acid (yellow) butt (A/A) indicate that the organism ferments both glucose and lactose. The organism ferments glucose, producing acid products. Once the glucose is consumed, it ferments lactose, breaking it down into glucose and galactose, causing the pH in the slant portion to remain acidic.
3. Alkaline (red) slant and alkaline (red) butt (K/K) indicate that the organism cannot ferment glucose or lactose and therefore produces no acidic products. The slant may become redder because of peptone catabolism. Often, with the lack of glucose fermentation, the butt is the same color as the butt of an uninoculated tube. Therefore some sources prefer the designation “no change” (NC) as opposed to alkaline.

If there are gas bubbles in the butt, splitting of the medium, or displacement of the medium from the bottom of the tube, the organism is aerogenic; that is, it can produce CO₂ and hydrogen gases during fermentation. This designation is noted in the shorthand designation as “g” (e.g., A/Ag). Any blackening in the butt indicates that the organism produces H₂S gas from thiosulfate. The H₂S combines with iron salt to produce ferrous sulfide, a black precipitate. H₂S is added after the slant/butt shorthand to signify that the organism produces H₂S (e.g., K/A H₂S).

With an inoculating needle that has a sample of a pure culture of the isolate, inoculate the medium by stabbing the butt and streaking the slant. The cap should be slightly loose. If the cap is screwed on too tightly, there will not be sufficient oxygen for peptone catabolism and, as a result, gram-negative

rods able to ferment only glucose may appear as lactose fermenters. Reactions should be interpreted at 18 to 24 hours. If the medium is read earlier, organisms able to ferment only glucose may appear to be lactose fermenters. If the medium is read later, lactose fermenters may consume the lactose and begin to catabolize peptones, with the slant reverting to red. A yellow slant and red butt may indicate failure to stab the butt or inoculation of the medium with gram-positive organisms. Examining the medium for a stab line or performing a Gram stain should clarify this situation.

The H₂S indicator system in KIA is not as sensitive as that found in other media, such as sulfide indole motility agar. A black butt should be read as acid, even though the yellow color may be obscured. If H₂S is reduced, this indicates that an acid condition does exist and can be assumed. Critical to understanding how this medium works is the fact that glucose is present in 10% the amount of lactose. Organisms use the simplest carbohydrate, glucose, first; once this is consumed, the organisms attack the more complex carbohydrate, lactose. If the organisms lack the appropriate enzymes, they move on to protein catabolism. There is sufficient lactose in the medium to prevent breakdown of peptone, provided it is read at the appropriate time.

Lim broth

Lim broth is used for the isolation of *Streptococcus agalactiae*, usually from vaginal or rectal swabs obtained from pregnant women who are being screened for group B streptococci carriage before delivery. Lim broth is a modified Todd-Hewitt broth. The Lim broth contains peptones, yeast extract, and glucose to support the growth of streptococci. Colistin and nalidixic acid are added to inhibit the growth of gram-negative organisms.

Lim broth is inoculated with the vaginal or rectal swab and incubated at 35°C with cap loosened for 18 to 24 hours. At this time, the broth is subcultured to a blood agar plate and then incubated at 35°C in 5% CO₂ for 24 hours. The plate is then examined for the presence of β-hemolytic colonies. If no such colonies are present after the first incubation, the plate should be reincubated and reexamined after another 24-hour incubation period.

Loeffler medium

Loeffler agar slant is used primarily for the recovery and identification of *Corynebacterium diphtheriae*. This medium contains beef serum, heart muscle infusion, and peptic digest of animal tissue. It can be used for the primary recovery of *C. diphtheriae* from nose and throat specimens and for subculture purposes. Because Loeffler medium is so enriched, *C. diphtheriae* grows well within 12 to 16 hours and produces non-distinctive translucent to gray-white colonies. The medium promotes the development of characteristic metachromatic granules that can be observed microscopically with methylene blue stains. High serum content and the incorporation of eggs enables the detection of proteolytic activity. Positive organisms produce colonies surrounded by small holes containing liquefied medium. The entire slant may eventually turn to liquid and produce a foul odor.

Loeffler serum slant should be inoculated as soon as possible after specimen collection, and selective media containing tellurite should always be used as well. Smears for *C. diphtheriae* should be prepared and examined after 8 to 24 hours of incubation. Although granule formation is typical of *Corynebacterium* spp., other organisms can produce a similar microscopic appearance. Therefore additional testing must be performed for confirmation of this organism.

Löwenstein-Jensen medium

Löwenstein-Jensen (LJ) medium is used to cultivate *Mycobacterium* spp. The potato flour, egg, and glycerol included in LJ medium help detoxify this medium and also supply nutrients required for growth of these organisms. Asparagine is included for maximum production of niacin by certain *Mycobacterium* spp. The malachite green inhibits the growth of other bacteria that may be present in specimens.

LJ medium is good for 1 month if tightly capped to prevent moisture loss and stored at 4° to 6°C. LJ medium must be kept out of direct light because malachite green is light-sensitive. Decontaminated, digested, or untreated specimens are inoculated onto the medium and incubated in a CO₂ incubator (5% to 10% CO₂) for 6 to 10 weeks. It is important to leave caps loosened for proper gas exchange. LJ medium can be prepared as deep tubes to be used in semi-quantitative catalase testing to help identify the *Mycobacterium* spp. LJ medium with 5% NaCl may be prepared to aid in identifying rapid growers. This medium is the same as LJ except that 5 g of NaCl is added per 100 mL of medium. The additional salt allows for testing the ability of certain mycobacteria to tolerate and grow in the presence of a high salt concentration.

Löwenstein-Jensen medium (Gruft modification)

LJ medium (Gruft modification), also known as *Löwenstein-Gruft medium*, is an enriched selective medium for the isolation of mycobacteria. The Gruft modification makes this medium more selective through the addition of penicillin (50 U/mL) and nalidixic acid (35 µg/mL). This formulation also includes 0.05 µg/mL of ribonucleic acid, which increases the rate of mycobacterium isolation over the standard LJ formulation. In the Petran and Vera modification, cycloheximide, lincomycin, and nalidixic acid are added to make LJ more selective. Both modifications can permit gentler decontamination or digestion procedures.

Lysine iron agar

Lysine iron agar (LIA) measures three parameters useful in identifying species of Enterobacteriales: lysine decarboxylation, lysine deamination, and H₂S production. LIA contains lysine (amino acid), glucose (carbohydrate source), a small amount of protein, bromocresol purple (pH indicator), and sodium thiosulfate and ferric ammonium citrate (sulfur

source and H₂S indicator). A purple color denotes an alkaline environment, designated P. Also, an R is used for the Bordeaux red color.

Three lysine-use patterns are possible:

1. Alkaline (purple) slant and alkaline (purple) butt (P/P) indicate that the organism decarboxylates lysine but cannot deaminate it. Initially, the organism ferments glucose, causing production of acid and changing the indicator in the butt to yellow. The organism then decarboxylates lysine to produce cadaverine, an alkaline product, which causes the pH indicator to change from yellow back to purple.
2. Alkaline (purple) slant and acid (yellow) butt (P/A) indicate that the organism fermented the glucose but was unable to deaminate or decarboxylate the lysine.
3. A red slant and acid (yellow) butt (R/A) indicate that the organism deaminated lysine but could not decarboxylate it. The yellow butt is caused by glucose fermentation. The red slant results from the product of lysine deamination combining with ferric ammonium citrate and flavin mononucleotide to form a burgundy color on the slant.

Any blackening in the butt indicates production of H₂S from sodium thiosulfate. This gas reacts with ferric salt to produce the black precipitate, ferrous sulfide. This medium appears purple before use. LIA is inoculated by stabbing the butt twice and streaking the slant with an inoculating needle. The cap should be left slightly loose because oxygen is required for deamination detection. Reactions should be read after 18 to 24 hours of incubation at 35°C. The medium may be incubated for up to 48 hours if needed. LIA is not as sensitive as other media for H₂S detection. Typically, H₂S-producing *Proteus* spp. may appear negative. Also, *Morganella morganii* produces a variable lysine deamination reaction after 24 hours of incubation. This medium can only be used with organisms that can ferment glucose. LIA is not a replacement for the Moeller decarboxylase tests.

MacConkey agar

MacConkey agar (MAC) is a selective, differential, primary plating medium. It selects for Enterobacteriales and other gram-negative bacilli in the presence of mixed microbiota and differentiates them into lactose fermenters and nonlactose fermenters. Bile salts and crystal violet inhibit most gram-positive organisms but permit growth of most gram-negative rods.

Lactose serves as the sole carbohydrate source. Gram-negative rods that ferment lactose produce pink or red colonies, which may be surrounded by precipitated bile. Acid production from lactose fermentation causes the neutral red dye absorbed into the colonies to change to red and can also cause the bile salts to become insoluble. Nonlactose-fermenting, gram-negative bacilli produce colorless or transparent colonies. Plates are streaked for isolation and incubated in ambient air, not in a CO₂ incubator, for 18 to 24 hours at 35°C. Weak, slow, or late lactose fermenters can produce colorless colonies at 24 hours or appear slightly pink in 24 to 48 hours. Plates should not be incubated longer than 48 hours because this can lead to confusing results.

Some gram-negative bacilli may fail to grow on this medium, whereas with prolonged incubation, some gram-positive bacteria, such as *Enterococcus* spp., can produce tiny colonies. Room temperature incubation may enhance recovery of *Yersinia enterocolitica*. The agar concentration may be increased to prevent swarming of *Proteus* spp. A formulation of MAC without crystal violet has been used to help identify mycobacteria.

MacConkey sorbitol agar

MacConkey sorbitol agar contains the same components as MAC, except D-sorbitol is substituted for lactose. This medium has been used to screen for *E. coli* O157:H7, which does not rapidly ferment sorbitol. Plates should be incubated at 35°C for 24 hours. Sorbitol-negative colonies will appear colorless on this medium and indicate possible *E. coli* O157:H7. Most other clinical isolates of *E. coli* produce a pink to red color on this medium.

Malonate broth

Malonate broth is used in the identification of species of Enterobacterales, particularly *Salmonella*. The broth contains sodium malonate (the primary carbon source), small quantities of glucose and yeast extract (nutrients), bromothymol blue (pH indicator), various salts, and a buffering system. Organisms producing a Prussian blue color can use malonate as a carbon source. If they can use malonate as a carbon source, they will also use ammonium sulfate as a nitrogen source, thereby producing alkaline products that cause an increase in pH and a change in the color of the medium to blue. Organisms unable to use malonate as a carbon source usually fail to grow, and the medium stays green. Because malonate resembles succinate, it competitively binds to succinate dehydrogenase, which catalyzes the succinate to fumarate reaction in the Krebs cycle. The inhibition of this enzyme, coupled with the inability to use malonate as a carbon source, prevents growth of the malonate-negative organism.

Malonate broth should be inoculated from triple sugar iron agar, KIA, or broth culture. The inoculum should be light. Cultures should be incubated at 35°C, checked at 18 to 24 hours, and checked again at 48 hours for production of a blue color. Some organisms produce only small amounts of alkalinity. Any trace of blue should be considered positive. Comparison with an uninoculated tube may be useful. Production of a yellow color is a negative reaction and is probably caused by fermentation of the small amount of glucose in the medium.

Mannitol salt agar

Mannitol salt agar is a selective and differential primary culture medium useful in the recovery and identification of staphylococci from specimens containing mixed microbiota. A high salt concentration (7.5%) inhibits most gram-negative and gram-positive bacteria except *Staphylococcus* spp. *S. aureus* can ferment mannitol, the sole carbohydrate in the

medium, to produce acid products. This lowers the pH and changes the color of the pH indicator, phenol red, to yellow. Plates are streaked for isolation and incubated at 35°C for 24 to 48 hours in ambient air. Colonies of *S. aureus* typically appear yellow, surrounded by a yellow zone. Most other *Staphylococcus* spp. usually do not ferment mannitol and therefore produce reddish colonies that may exhibit a red surrounding zone because of peptone breakdown. However, *Staphylococcus capitis*, *Staphylococcus xylosus*, and other staphylococci are mannitol positive and produce yellow colonies.

Enterococcus may be able to grow on mannitol salt agar and weakly ferment mannitol. Differentiation is readily accomplished through a catalase test. With prolonged incubation, organisms other than staphylococci can begin to grow and ferment mannitol. Some strains of *S. aureus* may slowly ferment mannitol, so plates should not be discarded until after 48 hours of incubation. All colonies suggestive of *S. aureus* should be further tested for coagulase or with another acceptable procedure. Subculture to less selective agar is preferable before performing these tests.

Methyl Red Voges-Proskauer medium

Methyl red Voges-Proskauer (MRVP) medium broth is useful in distinguishing among members of the Enterobacterales. For example, *E. coli* is positive for methyl red and negative for Voges-Proskauer; *Enterobacter aerogenes*, *Enterobacter cloacae*, and *Klebsiella pneumoniae* show the reverse reactions. Members of the Enterobacterales can be divided into two groups based on how they metabolize glucose. One group produces large amounts of mixed acids (lactic, formic, succinic, and acetic). When methyl red is added to one of these cultures after incubation, a red color is produced because of the acidic pH. The other group produces predominantly neutral end products, acetoin or acetylmethylcarbinol, by the butylene glycol pathway. When α -naphthol and 40% potassium hydroxide are added to the broth culture containing a member of this latter group, acetoin (if present) is oxidized to diacetyl in the presence of air and base. α -Naphthol catalyzes a reaction between the diacetyl and guanidine components of peptone to produce a pink-red color.

For the methyl red test, the broth culture is inoculated with a light inoculum and must be incubated for 48 hours. After incubation, the broth is divided into two tubes. The Voges-Proskauer test was originally designed to be performed after 5 days of incubation at 30°C. By using 0.5 to 1 mL of broth per tube, the test can be done after 18 to 24 hours of incubation at 35°C. Shaking aerates the broth culture and enhances the reaction. Enterobacterales are typically positive for methyl red or Voges-Proskauer, but not both.

Middlebrook 7H10 and 7H11 agars

Middlebrook 7H10 and 7H11 agars are used to cultivate *Mycobacterium* spp. Isoniazid-resistant strains grow better on these media, especially Middlebrook 7H11, than on egg-based media such as LJ medium. The Middlebrook agars are also more chemically defined than the LJ formulations. Middlebrook 7H10 and 7H11 are similar, except 7H11 contains

casein hydrolysate, which stimulates the growth of drug-resistant *Mycobacterium tuberculosis*. Both media contain growth factors, such as amino acids, glycerol, and inorganic salts that encourage recovery of mycobacteria. In addition, both formulations include OADC (oleic acid–dextrose catalase) enrichment, which chemically simulates egg components. The oleic acid is a fatty acid used by the mycobacteria. Albumin is added to inhibit toxic agents that might be present and to provide a source of protein. The added dextrose (glucose) is used by the mycobacteria to generate energy. The exogenous catalase neutralizes toxic peroxides. Malachite green is also added, although at a lower concentration than in LJ, and it gives some selectivity to the medium. Incubation in an aerobic atmosphere enriched with CO₂ is recommended.

Mitchison 7H11 selective agar

Mitchison 7H11 selective agar is prepared by adding antimicrobial agents to the Middlebrook 7H11 formulation, thereby making the medium more selective for mycobacteria. Amphotericin B, carbenicillin, polymyxin B, and trimethoprim are typically incorporated to inhibit gram-negative bacilli in particular, as well as yeast.

Motility test medium

The purpose of motility test medium is to determine whether an organism is motile or nonmotile. This test is particularly useful to identify members of the Enterobacterales in which two genera, *Shigella* and *Klebsiella*, are always nonmotile, and certain *Yersinia* spp. show motility at room temperature but not at 35°C. The nonglucose-fermenting, gram-negative bacilli can also be differentiated based in part on their motility. Furthermore, *L. monocytogenes* gives a classic, umbrella-shaped motility pattern below 30°C.

Nonmotile organisms, which lack flagella, grow only along the stab line, and the surrounding medium remains clear. Motile organisms, which usually possess flagella, move out from the stab line, and the medium appears turbid. A low agar concentration makes the medium semi-solid and permits better detection of motility. The medium is inoculated using an inoculating needle to stab the middle of the medium straight down and up once, without going all the way to the bottom of the medium. It is important to be careful and remove the inoculating needle along the initial stab line. Motility media should be incubated at 35°C. Because flagellar protein is not formed as well at higher temperatures, incubation at 20°C to 22°C is recommended. For *Yersinia* and *Listeria* species, noting the motility reaction at room temperature is particularly useful.

Triphenyltetrazolium chloride (TTC) may be added to the basic motility medium to enhance detection of motility. A 1% solution of TTC is prepared and filter-sterilized, and 5 mL of this solution is added to 1 L of motility medium. If TTC is used, bacteria incorporate colorless TTC and reduce it to a red formazan pigment. The medium shows reddening where there is growth. Other media such as sulfide indole motility (SIM) and motility indole ornithine (MIO) can be used to detect motility in addition to other reactions.

Mueller-Hinton agar

Mueller-Hinton agar is a transparent medium used in susceptibility testing of organisms to antimicrobial agents. Because Mueller-Hinton agar contains animal infusion, casein extract, and starch, it supports the growth of most organisms. In addition, sheep blood may be added to the basic formulation to perform susceptibility testing on streptococci, in particular *Streptococcus pneumoniae*. The addition of heated or chocolate sheep red blood cells to Mueller-Hinton agar makes an enriched medium that can be used for susceptibility testing of fastidious organisms, such as *Haemophilus* and *Neisseria*. Muller-Hinton agar also can be used for the X and V Factor test for *Haemophilus* spp. Starch is included in the medium for two reasons: it may protect the organisms against toxic substances and it can also serve as an energy source for some bacteria. Ca²⁺ and Mg²⁺ concentrations are critical in the testing of *Pseudomonas* isolates with aminoglycoside antibiotics. Usually, Mueller-Hinton agar contains enough bivalent cations, but it may be necessary to add these substances to Mueller-Hinton broth.

Mueller-Hinton agar with 2% NaCl

The addition of 2% NaCl to the basic Mueller-Hinton medium results in a medium that is selective for staphylococci. This formulation is used to detect methicillin-resistant *Staphylococcus aureus* (MRSA). The slower-growing resistant cells can be missed in a mixed population with methicillin-sensitive strains. Cefoxitin or oxacillin using the Kirby-Bauer method or Etest is useful in detecting MRSA. Once inoculated, the medium should be incubated at 30° to 35°C up to 48 hours to detect the heteroresistant methicillin-resistant isolates more accurately.

Mueller-Hinton agar with 4% NaCl and 6 µg/mL oxacillin

For this variation, 4% NaCl and 6 µg/mL oxacillin are added to the basic Mueller-Hinton medium to screen *S. aureus* isolates selectively for resistance to oxacillin or nafcillin. The inoculated plate should be incubated for 24 hours at 35°C in ambient air and examined using transmitted light for the presence of colonies.

Mycobactosel L-J medium

Mycobactosel, a BBL trade name, is a selective medium for the isolation of mycobacteria from specimens containing mixed biota. The medium contains cycloheximide, lincomycin, and nalidixic acid to inhibit contaminating organisms. Cycloheximide controls the growth of saprophytic fungi, while lincomycin inhibits gram-positive bacteria. Growth of some contaminating gram-negative bacteria encountered in clinical specimens is inhibited by nalidixic acid. The medium contains egg, glycerol, asparagine, malachite green, and other

various inorganic salts and nitrogen sources to support the growth of mycobacteria. Glycerol is a source for carbon and energy; asparagine promotes growth, and egg yolk is a source of lipids. Malachite green partially inhibits growth of contaminating bacteria.

New York City medium

New York City (NYC) medium is primarily used to isolate *N. gonorrhoeae* and *N. meningitidis* from specimens containing mixed normal microbiota. This medium will also support the growth of some mycoplasmas as well as *Ureaplasma urealyticum*. NYC medium is enriched with hemoglobin from lysed horse erythrocytes, yeast dialysate, and horse plasma. Selectivity for *N. gonorrhoeae* and *N. meningitidis* is accomplished by incorporating four antimicrobial agents that inhibit normal biota. Vancomycin prevents the growth of gram-positive bacteria, colistin inhibits gram-negative bacilli, and amphotericin B prevents the growth of yeast and molds. Trimethoprim is included to prevent swarming of *Proteus* spp. The concentrations of these antimicrobial agents are lower compared with MTM agar. Inoculated NYC plates should be incubated in increased CO₂ for several days. Also, a nonselective agar, such as CHOC agar, should be inoculated because 5% of gonococci are inhibited by the antimicrobial agents, particularly vancomycin and trimethoprim, found in this medium.

Nitrate broth

Nitrate broth is used to determine whether an organism can reduce nitrate to nitrite or to gaseous end products, such as nitrogen. The test is useful in identifying members of Enterobacterales and differentiation of nonglucose-fermenting, gram-negative rods, *Neisseria* spp., and *Moraxella catarrhalis*. The nitrate broth is inoculated with the test organism and incubated for 18 to 24 hours at 35°C.

The determination of nitrate reduction is performed in two parts. Sulfanilic acid and *N,N*-dimethyl-1-naphthylamine reagents are added first. If nitrate has been reduced to nitrite, nitrite will react with these reagents to form a red diazonium dye, *p*-sulfobenzene-azo- α -naphthylamine, and the test is positive. If there is no color change, then nitrate was not reduced or the nitrate was reduced to nitrogen gas. In this situation, zinc dust is added. Zinc reduces the remaining nitrate to nitrite, forming a red color. If nitrates are still present and a red color appears after the addition of zinc, then the organism is nitrate reduction-negative. However, if nitrate was reduced to nitrogen gas, no color change occurs, and the test is interpreted as positive. The presence of gas can be detected by putting a Durham tube into the broth before incubation. Gas produced during nitrate reduction will be captured in the inverted tube and seen as a bubble.

The results should be determined immediately after the addition of the reagents because the color fades quickly. If it is necessary to add zinc, avoid using large amounts. Too much zinc can result in the formation of hydrogen gas, which can cause reduction and decrease the color reaction. The medium may need to be supplemented with serum and incubated for

up to 5 days when testing *Neisseria* spp. An uninoculated control tube using the reagents should be performed alongside the patient test tube to ensure that glassware, reagents, and supplies are nitrate free.

Nutrient agar

Nutrient agar has been used to distinguish between the non-fastidious, less pathogenic *Neisseria* spp. and pathogenic *Neisseria* spp., such as *N. gonorrhoeae* and *N. meningitidis*. Nutrient agar contains minimal nutrients and an especially low concentration of protein. Growth of an isolate on this medium means that it is not fastidious and does not require special supplements. The less fastidious neisseriae grow on nutrient agar, whereas the more pathogenic species do not. Nutrient agar has also been used for the maintenance of stock cultures.

Oxidative-fermentative medium (Hugh and Leifson formulation)

Oxidative-fermentative (OF) medium is used to determine whether a gram-negative, nonglucose-fermenting rod is oxidative, fermentative, or biochemically inert. Three modifications over traditional media make this medium useful in testing nonfermenting gram-negative bacilli. A low concentration of peptone prevents the formation of alkaline products that can neutralize small quantities of acid produced through oxidation. The high concentration of carbohydrate increases the potential amount of acid that can be formed, and the lower concentration of agar makes the medium semi-solid, permitting acids formed on the surface to diffuse throughout the medium. Bromothymol blue serves as the pH indicator for acid detection.

The classic method for using this medium involves the stabbing of two tubes with the organism. The medium in one tube is covered with vaspar (a mixture of petrolatum and paraffin) or melted paraffin. Sterile mineral oil has been used for this purpose but is not recommended because it does not block oxygen as well. Tubes are incubated at 35°C. Several days of incubation may be required because of the slower growth of some nonfermenting gram-negative bacilli. A color change to yellow in both tubes means that the organism is fermentative (i.e., it can produce acid in the absence of oxygen). Color change to yellow in only the uncovered tube means that the organism is oxidative (requires oxygen to use the carbohydrate). If neither tube changes in color or the covered tube shows no change but the uncovered tube turns blue, the organism cannot use the carbohydrate oxidatively or fermentatively and is considered inert.

A one-tube modification of this test has been described. One tube is stabbed, and it is not covered. Color change to yellow only near the top of the medium indicates the oxidative use of glucose. If the entire tube changes to yellow, fermentation is suggested. Because the medium is semi-solid, motility may be observed in this medium. Sometimes OF medium is used for differentiating staphylococci (fermentative) from micrococci (oxidative). This testing requires a different formulation.

Oxidative-fermentative base, polymyxin B, bacitracin, lactose agar

Oxidative-fermentative base, polymyxin B, bacitracin, lactose agar (OFPBL) is a selective and differential medium used for the isolation of *Burkholderia cepacia* from respiratory samples of patients with cystic fibrosis. Polymyxin B and bacitracin are added to inhibit the growth of most gram-negative and gram-positive bacteria. The medium differentiates between colonies that grow based on the ability of the isolate to ferment lactose. *B. cepacia* can ferment lactose and appears as a yellow colony. Nonlactose-fermenting colonies that grow appear green. OFPBL plates should be inoculated with the specimen and incubated aerobically at 30°C.

Phenylalanine deaminase agar

Phenylalanine deaminase (PAD) agar is used to detect an organism's ability to deaminate phenylalanine. A positive reaction is most useful for distinguishing *Proteus*, *Providencia*, and *Morganella* spp. from other members of Enterobacterales. This test also can be used to distinguish phenylpyruvate-positive *Moraxella* organisms from other *Moraxella* spp.

PAD agar includes phenylalanine, yeast extract, a nitrogen and carbon source, various salts, and agar. Protein hydrolysates and meat extracts are not included because these substances contain a variable amount of phenylalanine. The PAD slant is inoculated with the test organism, and the slant is incubated at 35°C for 18 to 24 hours. At the end of the incubation period, a few drops of 12% FeCl₃ are added to the tube so they will run down the slant. If an organism produces phenylalanine deaminase, it converts phenylalanine to the α -keto acid, phenylpyruvic acid. This acid reacts with the ferric chloride reagent to form a dark green complex. The immediate appearance of a dark green slant on addition of ferric chloride reagent is a positive reaction; no color change on addition of reagent is a negative reaction.

Phenylethyl alcohol agar

Phenylethyl alcohol (PEA) agar is used primarily to isolate gram-positive cocci, such as staphylococci and streptococci, from specimens with mixed microbiota. Most gram-positive bacilli will also grow on this medium, except *Bacillus anthracis*, which is unique among the *Bacillus* spp. in its lack of growth on this medium.

PEA agar resembles sheep blood agar except that PEA contains phenylethyl alcohol. This component inhibits facultative gram-negative rods, especially swarming *Proteus* spp. PEA is volatile, and plates should be tightly sealed in plastic bags and stored in the refrigerator. Hemolytic reactions are not dependable on this medium because of the action of PEA on cell membranes. Gram-negative rods can grow on PEA agar, but colonies are smaller than usual and can be readily differentiated from those of gram-positive bacteria. *P. aeruginosa* is not inhibited by this medium. Some gram-positive cocci may require more than 24 hours of incubation to grow

well on PEA agar. An anaerobic formulation can be achieved by adding PEA to CDC anaerobic agar before autoclaving or by supplementing the formulation with hemin, vitamin K, and sheep blood after sterilization of basal medium. The anaerobic formulation of this medium selects for gram-negative and gram-positive obligate anaerobes while inhibiting facultatively anaerobic gram-negative bacilli.

Tellurite blood agar

Tellurite blood agar is a selective, differential, enrichment agar useful in isolating *Corynebacterium diphtheriae*. Animal blood or hemoglobin powder and vitamin growth supplement are added as enrichment. Some formulations also incorporate cystine to enhance the growth of fastidious organisms further, including *C. diphtheriae*. Potassium tellurite is the selective and differential ingredient responsible for inhibiting the growth of gram-negative organisms, staphylococci, and streptococci while allowing the growth of *C. diphtheriae* and diphtheroids. *C. diphtheriae* reduces the tellurite, resulting in a black colony.

Tubes of base medium can be stored and melted to make the complete medium as culture plates are needed. Freshly plated medium should be inoculated, streaked for isolation, and incubated at 35°C. On this medium, colonies of *C. diphtheriae* are dull gray-black, whereas diphtheroids are light gray-green with dark centers. Some *Staphylococcus* spp., gram-negative bacilli, and yeasts may overcome inhibition and grow on this medium. The *Staphylococcus* colonies are large, glistening, and jet black, whereas those of gram-negative bacilli and yeasts are dull gray-black but larger than the *C. diphtheriae* colonies.

PPLO agar

PPLO agar is used to isolate *Mycoplasma* spp. Its name is derived from the original name for *Mycoplasma*: a *pleuropneumonia-like organism*. Nutrients in this medium are provided by yeast-enriched peptones, serum, and heart infusion. NaCl is added to maintain proper osmotic pressure. Agar is added to solidify the medium, but at a lower concentration than in other solid agar plates. With less agar, the medium is softer so the mycoplasmas can grow into the medium and grow only slightly on top of the plate. Antimicrobials can be added to inhibit the growth of other bacteria. The medium should be inoculated with the specimen and incubated at 35°C in 5% to 10% CO₂. Plates should be examined under a stereomicroscope for the growth of small (0.01- to 0.5-mm diameter) colonies, some of which might have a dense center surrounded by a less dense periphery (fried egg-like). Plates should be saved for at least 7 days before they are discarded as negative.

Regan-Lowe medium

Regan-Lowe medium is enriched and selective for the isolation of *Bordetella pertussis* and *Bordetella parapertussis* from clinical specimens. The nutritional base is composed of beef

extracts, horse blood, niacin, and pancreatic digests. Charcoal and starch are added to neutralize inhibitors, especially fatty acids and peroxides that might be present in the medium. Cephalixin is the selective agent.

Inoculated plates should be incubated aerobically at 35°C in a moist environment for 5 to 7 days. *B. pertussis* colonies are domed, shiny, transparent, and tiny. The colonies are described as resembling mercury droplets. Regan-Lowe transport medium is also available. The transport medium differs from the isolation medium in that the transport medium uses lysed horse blood, whereas whole horse blood is used in the isolation medium. In addition, the transport medium contains one half as much charcoal as the isolation medium.

Salmonella-Shigella agar

Salmonella-Shigella (SS) agar is used to select for *Salmonella* and some strains of *Shigella* in stool specimens. SS agar is also differential in that these organisms produce characteristic colonies on the medium. SS agar contains bile salts, sodium citrate, and brilliant green, which inhibit the growth of gram-positive and many lactose-fermenting, gram-negative bacilli normally found in feces. Lactose is the sole carbohydrate source in the medium, and neutral red is the pH indicator. If an organism grows on the medium and ferments lactose, it will produce acid and change the indicator to pink-red. Sodium thiosulfate is added as a source of sulfur for the production of H₂S. If H₂S is produced, it reacts with the ferric ammonium citrate present in the medium, forming a black precipitate in the center of the colony.

A heavy inoculum of stool can be plated on SS agar because the formulation is so inhibitory; however, strains of *Shigella* may not grow on SS agar, and this medium should not be used as the sole primary plating medium when *Shigella* is a potential isolate. *Shigella* colonies appear colorless on SS agar because these organisms do not ferment lactose or produce H₂S. *Salmonella* colonies are colorless, with a black center, because these organisms usually make H₂S but do not ferment lactose. Pink to red colonies indicate that the organism ferments lactose; if there is a black center, it also produces H₂S. If *Proteus* grows on this medium, swarming is inhibited.

Schaedler agar

Schaedler agar is an enriched medium used for the isolation of anaerobic bacteria. Vegetable and meat peptones, yeast extract, and glucose provide the nutrients. The growth of more fastidious anaerobes is aided by the addition of vitamin K, sheep blood, and hemin. Facultative anaerobes also will grow on this medium, so aerotolerance testing should be performed on all isolated colonies to determine their oxygen dependency.

Selenite broth

Selenite broth is an enrichment broth used for the recovery of low numbers of *Salmonella* and some strains of *Shigella* from stool and other specimens containing large amounts of mixed

bacteria. The sodium selenite present in this medium inhibits the growth of many gram-negative bacilli and enterococci but permits the recovery of *Salmonella* and some *Shigella* species. Selenite is most effective at a neutral pH. Reduction of selenite during bacterial growth produces alkaline products that may inhibit the growth of salmonellae and also reduce the toxicity of the selenite for other organisms, so lactose and phosphate buffers have been included in this medium to maintain a neutral pH. Lactose fermenters produce acid, which neutralizes these alkaline products and returns the medium to a neutral pH.

Approximately 1 to 2 g of stool should be inoculated into the broth, which is then incubated at 35°C. The broth should be subcultured to enteric media after it has incubated for 12 to 18 hours (some references suggest 6 to 12 hours). Beyond this time frame, overgrowth with normal biota is likely because the inhibitory effect of the selenite decreases after 12 hours. A variation of the selenite broth formulation includes the addition of cystine to increase the recovery of *Salmonella*.

Sodium chloride broth, 6.5%

Sodium chloride broth is useful in the differentiation of streptococci. It primarily distinguishes *Enterococcus* spp. (positive) from group D streptococci (negative), both of which produce a positive bile-esculin reaction. In addition, viridans streptococci can be distinguished from *Enterococcus* (which may sometimes appear α -hemolytic) because the viridans streptococci, like group D streptococci, cannot grow in this medium.

Sodium chloride broth is prepared from heart infusion broth, a general-purpose medium that already contains 0.5% NaCl. NaCl is added to this medium to bring the salt concentration to 6.5%. Sodium chloride broth also contains glucose as a carbohydrate source, and some formulations add bromocresol purple, a pH indicator. If the organism can tolerate this high concentration of salt, it will grow in the medium and produce turbidity. Fermentation of glucose produces acid and can cause the medium to turn from purple to yellow if the pH indicator is present. Several colonies are inoculated into the broth and incubated overnight at 35°C. Any growth in the broth is considered positive, even if the indicator does not change color. To avoid a false-negative result, the broth should be gently mixed before interpretation. Inoculating the broth too heavily may give a false-positive result. Organisms other than enterococci, such as group B streptococci and aerococci, can produce positive results.

SP-4 broth and SP-4 agar

SP-4 broth and SP-4 agar serve as primary isolation media for *Mycoplasma* spp. SP-4 media contain yeast products that serve as growth factors for *Mycoplasma* and supply preformed nucleic acid. Fetal bovine serum supplies the cholesterol necessary for the synthesis of sterols for the bacterial membranes. Antimicrobials inhibit normal biota that may be present in the specimen. Penicillin is included to prevent the growth of gram-positive bacteria, amphotericin B inhibits fungi, and

polymyxin B inhibits gram-negative bacilli. Biphasic media provide microaerophilic and moist conditions, which some *Mycoplasma* spp. prefer.

Streptococcus-selective agar

Streptococcus-selective agar is a selective medium used to isolate streptococci, primarily to detect β -hemolytic streptococci in throat swabs. Columbia agar is the base to which ribonucleic acid and maltose are added to enhance the production of streptolysin S. Polymyxin B and neomycin are added to inhibit the growth of gram-positive and gram-negative organisms that are found as normal oral biota. Another formulation incorporates colistin and oxolinic acid to suppress the normal biota. Nonselective media (e.g., a blood agar plate) also should be inoculated with the specimen, and the growth on both plates should be compared.

Tetrathionate broth

Tetrathionate broth is an enrichment medium used for recovery of *Salmonella*, except serotypes Typhi and Arizonae, from stool specimens. Tetrathionate is produced when an iodine-potassium iodide solution is added to the basal broth. Bile salts in conjunction with thiosulfate and the iodine-iodide solution inhibit the growth of most gram-negative and gram-positive organisms, except *Salmonella*. Some formulations also include brilliant green or crystal violet, which increases the inhibitory nature of the medium. The medium must be used within 24 hours of preparation. Because the basal medium may be stored in the refrigerator indefinitely, some microbiologists prefer to dispense 10 mL of basal medium per tube. Just before use, 0.2 mL of iodine solution can be added to each tube. A heavy inoculum of stool can be added to the broth. After 12 to 24 hours of incubation at 35°C, the broth should be subcultured to enteric media to prevent overgrowth with normal biota. This medium inhibits most *Shigella* spp.

Thayer-Martin, modified agar

MTM agar is a selective enrichment medium used for recovering *N. gonorrhoeae* and *N. meningitidis* from specimens that have mixed microbiota. MTM agar is highly enriched to support the growth of the more fastidious *Neisseria* spp. A chocolate agar base and added growth factors such as hemoglobin, vitamins, diphosphopyridine nucleotide, L-cysteine, NAD, and glutamine are the main constituents of MTM. Corn starch also is included to absorb inhibitory substances that might be present. The modified formulation has less agar and glucose than the original Thayer-Martin medium. These changes seem to result in improved recovery of *Neisseria* spp.

MTM and Thayer-Martin media both contain antimicrobial agents that allow for the growth of pathogenic *Neisseria* spp. and prevent the growth of most other organisms. Both media contain vancomycin to inhibit the growth of gram-positive bacteria, colistin to inhibit gram-negative bacilli, and nystatin to prevent the growth of fungi. MTM differs from the original Thayer-Martin formulation by the addition of trimethoprim, which prevents *Proteus* spp. from swarming.

Martin-Lewis agar is a modification of the MTM formulation. In Martin-Lewis agar, anisomycin (20 μ g/mL) is substituted for nystatin as the antifungal agent. In addition, the vancomycin concentration (4 μ g/mL) is higher than in the MTM formulation. MTM plates should be incubated in a CO₂ incubator for several days. Because some strains of *N. gonorrhoeae* can be inhibited by vancomycin and trimethoprim, a CHOC plate should be used in conjunction with an MTM plate.

Thioglycollate broth, basal and enriched

Thioglycollate broth is an all-purpose medium that can be used to isolate a wide range of bacteria. It is often used along with culture plates when inoculating clinical specimens. In this case, it helps detect organisms present in low numbers, such as anaerobes, that are present in the original specimen. Formulations without glucose can be used in fermentation studies of anaerobes.

Nutrients are provided by the incorporation of casein, cysteine, glucose, and yeast extract. Thioglycollate, cystine, and sodium sulfite act as reducing agents in this medium, and the low concentration of agar prevents downward diffusion of oxygen, allowing for the growth of anaerobic organisms toward the bottom of the tube. Various supplements can be added for enrichment to support the growth of more fastidious organisms. These include hemin (5 μ g/mL), vitamin K (0.1 μ g/mL), and sodium bicarbonate (1 mg/mL), which can be autoclaved in the medium. Supplements that must be added after autoclaving include rabbit or horse serum (10% vol/vol) and Fildes enrichment (5% vol/vol), which are given as final concentrations in the medium. Thioglycollate broth should be stored at room temperature and then boiled and cooled before use. After inoculation of the clinical specimen, the medium is incubated at 35°C for 3 to 7 days and examined for turbidity. Smears for Gram stain should be made and compared with growth obtained on primary culture plates. If something different appears to be growing in the broth, subcultures should be performed. Bile salts can be added to make thioglycollate bile broth select for the growth of *Bacteroides fragilis* and *Clostridium perfringens* from clinical specimens.

Thiosulfate citrate bile salts sucrose agar

Thiosulfate citrate bile salts (TCBS) sucrose agar is a selective medium used to isolate *Vibrio* spp. from stool specimens. TCBS agar can also differentiate *Vibrio* spp. based on sucrose utilization. *Vibrio* spp. grow poorly on media designed for isolation of *Salmonella* and *Shigella* but produce colorless colonies on MAC. TCBS agar includes sodium citrate, sodium thiosulfate, and oxgall (10% solution equivalent to full-strength bile), which together inhibit many gram-positive cocci and gram-negative bacilli normally present in stool specimens. In addition, the high pH of TCBS agar encourages the growth of *Vibrio* spp. while inhibiting other organisms.

The ability of an organism to ferment sucrose is the basis for differentiating among various species of *Vibrio*. Bromothymol blue and, in some formulations, thymol blue are incorporated as pH indicators. *Vibrio cholerae* and *Vibrio alginolyticus* produce yellow colonies because they can ferment sucrose, whereas *Vibrio parahaemolyticus* and *Vibrio vulnificus* usually produce blue-green colonies because they do not ferment sucrose. Organisms that can produce H_2S from sodium thiosulfate have black centers because of the reaction of this gas with ferric citrate. *Vibrio* spp. do not produce H_2S . Oxgall and sodium cholate or bile salts (8 g/L) can be used in place of oxgall alone.

When using TCBS agar, a heavy inoculum should be applied because *Vibrio* spp. die quickly, and this medium is very inhibitory. A fresh specimen is best because these organisms are sensitive to dry conditions, sunlight, and acid pH. If there is a delay in plating, Cary-Blair semi-solid transport medium should be inoculated with the stool specimen rather than buffered glycerol transport medium. Plates should be incubated at 35°C for 18 to 24 hours and up to 48 hours. Oxidase testing cannot be performed on colonies isolated on TCBS agar. Occasionally, strains of *V. cholerae* produce blue-green colonies on this medium because of delayed sucrose fermentation. Some *Vibrio* spp. do not grow well on this medium. Also, other organisms, such as *Pseudomonas*, *Plesiomonas*, and *Aeromonas*, can grow on TCBS agar and usually produce blue colonies; therefore these must be distinguished from *Vibrio* spp.

Tinsdale agar

Tinsdale agar is a selective differential medium useful in isolating and identifying *C. diphtheriae* from specimens containing mixed biota. Tinsdale agar contains a high concentration of potassium tellurite, which inhibits the growth of most normal biota but permits *Corynebacterium* spp., especially *C. diphtheriae*, to grow. All *Corynebacterium* spp. growing on the medium produce gray to black colonies because of the reduction of tellurite to tellurium. In addition, *C. diphtheriae* colonies are surrounded by a brown halo. The brown halo is produced from the interaction of tellurite with the H_2S produced by the organism from cystine and thiosulfate. The basal medium can be stored indefinitely; tellurite and serum can be added just before use. Once prepared, the medium has a shelf life of 4 days.

When using Tinsdale agar, the plates are streaked for isolation, and the medium is stabbed in several areas. Sometimes browning occurs in these stabbed areas before it can be seen around surface colonies. Plates should be incubated at 35°C for 24 to 48 hours in ambient air. Increased CO_2 can slow down the production of the brown halo. It may require 48 hours for some *C. diphtheriae* strains to produce the characteristic halo. In addition, *Corynebacterium ulcerans* and *Corynebacterium pseudodiphtheriticum* also may produce a dark halo on this medium and must be differentiated from *C. diphtheriae*. Other organisms occasionally grow on Tinsdale agar. *Proteus* produces mucoid colonies and tends to blacken the medium. Rare streptococci and staphylococci can produce dark colonies with a surrounding halo but may be distinguished from the corynebacteria by performing a Gram stain.

Todd-Hewitt broth with gentamicin and nalidixic acid

Todd-Hewitt broth is used to grow streptococci for serotyping. Nutrients are provided by peptones and beef heart infusion, and glucose is added as an energy source. Throat swabs can be used to inoculate the broth that is then incubated at 35°C for 2 to 5 hours for serologic testing for detection of group A streptococci. The broth can be incubated for up to 24 hours then streaked for isolation on SBA plates. Pure cultures of β -hemolytic streptococci can be inoculated into the broth prior to serologic testing.

The selective formulation can be used to isolate group B streptococci from vaginal and rectal swabs. It can be used, however, to isolate many β -hemolytic streptococci from a clinical specimen that has mixed biota. Colistin and nalidixic acid (Lim broth) or gentamicin and nalidixic acid (Trans-Vag broth) can be added to inhibit the growth of gram-negative bacilli and select for group B streptococci. This broth is inoculated with the vaginal or rectal swab and incubated at 35°C for 18 to 24 hours. The broth is then subcultured to a blood agar plate, incubated for 24 hours at 35°C in 5% CO_2 , and examined for the presence of β -hemolytic colonies.

Triple sugar iron agar

Triple sugar iron (TSI) agar can be used to determine whether a gram-negative bacillus is a glucose fermenter or non-glucose fermenter, a fundamental characteristic in the initial classification of gram-negative rods. The medium also tests for sucrose and/or lactose fermentation, gas production during glucose fermentation, and H_2S production, all of which are useful in the differentiation of gram-negative rods belonging to the Enterobacteriales.

TSI agar contains glucose, sucrose, and lactose (fermentable carbohydrates); sucrose and lactose are each present at 10 times the glucose concentration. Other components include phenol red (pH indicator), peptone (carbon/nitrogen source), and sodium thiosulfate plus ferric ammonium sulfate (sulfur source and H_2S indicator, respectively). TSI agar resembles KIA except that TSI contains sucrose. Carbohydrate fermentation patterns resemble those of KIA; however, an acid (yellow) slant and acid (yellow) butt (A/A) in the TSI indicate that an organism ferments glucose and sucrose and/or lactose. Gas bubbles indicate gas produced from fermentation, and blackening indicates H_2S production, the same as in KIA. Precautions concerning the inoculation and incubation outlined in the KIA description also apply to TSI.

Trypticase soy agar

Trypticase soy agar (TSA) is an all-purpose medium that supports the growth of many organisms. It is frequently used as the basal medium for sheep blood agar plates. TSA contains peptones from soybeans and casein as a nutrient source and NaCl as an osmotic stabilizer. Agar serves as a solidifying agent. Unlike trypticase soy broth (TSB), TSA does not contain

glucose, which can interfere with the expression of β -hemolysis on sheep blood agar plates. Formulation with sheep blood and gentamicin added is used for the recovery of *Streptococcus pneumoniae*. Another formulation contains sheep blood and vancomycin to recover vancomycin-resistant enterococci.

Trypticase soy broth

TSB is an all-purpose medium that supports the rapid growth of most organisms, including streptococci, without added supplements. TSB contains soybean and casein digests as protein sources, NaCl for osmotic stability, glucose, and dipotassium phosphate as a buffer. Glucose fermentation can lower the pH causing acid-sensitive organisms such as *S. pneumoniae* to die at 24 hours of incubation. This broth is used as a medium in some blood culture bottles, sometimes to make an inoculum for Kirby-Bauer susceptibility testing, and as a medium for sterility testing. It should not be used for primary inoculation of clinical specimens.

Tryptophan broth, 1%

Tryptophan broth is used for performing the indole test, a procedure that is particularly useful in identifying species of Enterobacterales and identifying nonglucose-fermenting, gram-negative bacilli. This broth contains trypticase, a peptone rich in tryptophan, and NaCl, which serves as an osmotic stabilizer. Some bacteria possess the tryptophanase enzyme system, which hydrolyzes and deaminates tryptophan, producing indole, pyruvic acid, and ammonia. When Ehrlich or Kovac reagent is added to a tryptophan broth culture, indole produced by the organism reacts with the aldehyde portion of dimethylaminobenzaldehyde, the primary chemical in these reagents, and forms a red color.

Tryptophan broth should be inoculated with a few colonies from a pure culture and incubated for 24 hours at 35°C. Five drops of Kovac reagent are added to the medium; a red color in the reagent layer or at the interface of the reagent and broth signifies the presence of indole (positive). If Ehrlich reagent is used, 1 mL of xylene is first added to the broth culture at the end of the incubation period to extract indole. The tube is shaken, and five drops of the reagent are added. Kovac reagent is generally used when testing members of Enterobacterales, and Ehrlich reagent is used when testing nonglucose-fermenting, gram-negative bacilli and anaerobes. Other media have been developed to detect the production of indole from tryptophan: sulfide indole motility (SIM) agar, indole nitrate broth, and motility indole ornithine (MIO) medium. A rapid spot test using filter paper saturated with para-dimethylaminocinnamaldehyde reagent also has been used for indole determination. In this test, the organism is rubbed onto the surface of filter paper saturated with the spot indole reagent; the appearance of a blue color indicates a positive test.

Urea agar and broth

Urea media detect an organism's ability to hydrolyze urea. This is particularly useful in identifying species of

Enterobacterales. In these media, urea is hydrolyzed by the enzyme urease to form CO₂, water, and ammonia. The ammonia then reacts with components in the medium to form ammonium carbonate. This compound causes an increase in pH, which changes the pH indicator, phenol red, to pink. Neither the agar nor the broth formulations contain much protein, which prevents the formation of alkaline products from the breakdown of peptones and could result in false-positive results. The broth formulation contains monopotassium phosphate and disodium phosphate, which make the medium highly buffered. In addition, the broth formulation lacks glucose and peptone. Only organisms that are strong urease producers and not fastidious (e.g., *Proteus* spp.) will appear positive in this type of medium. The agar formulation is less buffered, so smaller amounts of urease activity can be detected. Also, glucose and peptone, which help support growth, are included.

The agar slant is inoculated with an inoculating loop or needle that has a small amount of the organism and incubated at 35°C for 18 to 24 hours. If urease is produced, the medium will turn pink. Rapid urease producers such as *Proteus* spp. turn the entire tube pink and may be detectable in a few hours. Slow urease producers, such as *Klebsiella*, only turn the slant pink. If the organism does not produce urease, there will be no color change. Stuart urea broth is incubated at 35°C for 18 to 24 hours. A positive reaction is red color throughout the broth.

Vaginalis agar

Vaginalis (V) agar is a selective or nonselective (depending on the addition of antimicrobial agents) enrichment medium useful in the isolation of *Gardnerella vaginalis*. V agar is a Columbia agar base with added peptone to provide nutrients, corn starch as an energy source, and human blood, rather than sheep blood, to detect hemolysis. *G. vaginalis* produces diffuse β -hemolysis only on media containing human blood. V agar can be made selective by the addition of colistin, nalidixic acid, and nystatin, which will inhibit many of the organisms that are found as normal vaginal biota.

Inoculated V agar plates are incubated in 5% CO₂ at 35°C. Plates can be observed at 24 hours, but often 48 to 72 hours is required for *G. vaginalis* to grow. The organism produces tiny dome-shaped, white colonies surrounded with zones of diffuse β -hemolysis.

Xylose-lysine-deoxycholate agar

Xylose-lysine-deoxycholate (XLD) agar is a selective, differential, primary plating medium used to isolate *Salmonella* and *Shigella* spp. from stool and other specimens containing mixed biota. *Salmonella* and *Shigella* spp. produce characteristic colonies on XLD, which aids in their recognition. XLD agar contains sodium deoxycholate, which inhibits gram-positive cocci and some gram-negative bacilli found in feces as normal biota. Because XLD agar has a lower concentration of bile salts than other formulations of enteric media, such as SS and HE agars, it is less selective but permits better recovery of *Shigella*. XLD agar contains three fermentable

carbohydrates—sucrose and lactose, which are present in high concentration, and xylose, which is present in a lower concentration. Phenol red is the pH indicator. The amino acid lysine is included to detect lysine decarboxylation. Sodium thiosulfate is a sulfur source from which organisms can make H_2S . Ferric ammonium citrate in the medium combines with H_2S to produce ferrous sulfide, a black precipitate.

Four types of colonies are produced on XLD agar. Yellow colonies are formed by organisms such as *E. coli* that ferment the excess carbohydrates to produce a large amount of acid and change the pH indicator to yellow. Because there is excess carbohydrate, these organisms do not decarboxylate the lysine, even though they can possess lysine decarboxylase. Bacteria that ferment only xylose and do not decarboxylate lysine also produce yellow colonies. Yellow colonies with black centers are organisms that ferment the excess carbohydrate and produce H_2S . Examples of organisms that produce this colony type are *Citrobacter* and some *Proteus* spp. *Shigella* and *Providencia*, which neither ferment xylose, lactose, or sucrose nor produce H_2S , produce colorless or red colonies. *Salmonella* and *Edwardsiella* produce red colonies with black centers. *Salmonella* and *Edwardsiella* ferment xylose to make acid, producing a yellow colony, but because xylose is present in limiting concentrations and is consumed, these organisms

will then decarboxylate the lysine to produce cadaverine, an alkaline product that causes the colony to revert to red. Blackening is caused by H_2S production.

XLD agar should be incubated at 35°C for 24 hours in ambient air. Some investigators have recommended incubating plates for up to 48 hours to enhance blackening in *Salmonella* colonies. With prolonged incubation, the delicate balance of this medium can be altered, and distinguishing normal biota from potential pathogens becomes more difficult. *Shigella dysenteriae* and *Shigella flexneri* can occasionally be inhibited on XLD agar. Some strains of *Salmonella* may fail to produce H_2S and therefore resemble *Shigella* colonies. On this medium, blackening is more likely to occur when alkaline conditions exist.

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Selected mycology culture media and stains

Connie R. Mahon and Donald C. Lehman

Fungal media

A variety of enrichment and selective media is available to the clinical laboratory for the isolation and identification of pathogenic fungi. This appendix provides information about a select number of mycology media. Most media discussed in this appendix are commercially available in dehydrated or finished form. Recommendations regarding general use of the media, enrichment or selective, or combinations of each type, are outlined in Chapter 27 of this text.

Antifungal susceptibility testing media

The M27 and M38 Clinical and Laboratory Standards Institute (CLSI) documents describe the procedures for determining the susceptibility of yeasts and filamentous fungi to antifungal agents by broth dilution. Documents M44 and M60 describe antifungal disk diffusion and performance standards. The recommended medium for broth dilution is RPMI 1640 medium without sodium bicarbonate but with L-glutamine and glucose. 3-*N*-morpholinopropanesulfonic acid (MOPS) is added as a buffer to maintain the pH of the medium at 7.0. Yeast nitrogen base broth with 2% glucose also can be used for broth dilution procedures. Mueller-Hinton agar with 2% glucose and 0.5 µg/mL methylene blue dye is the recommended medium for disk diffusion testing.

Assimilation base for carbohydrates

Modified yeast nitrogen base is a synthetic basal medium that provides a sufficient source of nitrogen to support the growth of fungi during carbohydrate assimilation testing. Fungal isolates are plated for confluent growth, and carbohydrate disks are dispensed onto the surface to provide the specific carbohydrates for assimilation testing. The plates are incubated at 30°C for 48 to 72 hours and examined for growth around each disk. Good growth around a disk indicates assimilation

of that carbohydrate, whereas scant or no growth around a disk indicates no assimilation. Glucose (dextrose), used by all yeasts, serves as the growth control.

Alternatively, the basal media can be prepared as a broth to which individual carbohydrates are added in separate tubes. The yeast is inoculated into the series of tubes. The tubes are incubated, and the broth will become turbid if the yeast can assimilate the carbohydrate. The pattern of carbohydrate assimilation is used to identify yeasts.

Birdseed agar

Birdseed agar is a selective and differential enrichment medium designed for the isolation and preliminary identification of *Cryptococcus neoformans* and *Cryptococcus gatti*. The ground seeds (niger seeds) of *Guizotia abyssinica* provide enrichment as well as caffeic acid, which provides a substrate for the detection of phenol oxidase activity. On this medium, *C. neoformans* and *C. gatti* colonies typically display a tan to rich brown color because phenol oxidase activity results in the deposition of melanin in yeast cell walls. The colonies of other yeasts remain cream-colored. Chloramphenicol can be added to inhibit the growth of bacteria.

Bismuth sulfite–glucose–glycine yeast agar

Bismuth sulfite–glucose–glycine yeast (BiGGY) agar is a selective and differential medium for the isolation and differentiation of *Candida* spp. from clinical specimens. BiGGY contains yeast extract, glucose, glycine, bismuth ammonium citrate, and sodium sulfite. Yeast extract and glucose provide nutrients for growth. Glycine in high concentration is an additional nutrient and also inhibits many bacterial species. *Candida* spp. reduce bismuth salt to bismuth and sulfite to sulfide. Bismuth and sulfide combine to form a brownish to black precipitate that colors the colonies and also diffuses into the medium. *Candida albicans* forms brown to black colonies with no diffusible pigment. *Candida tropicalis* produces dark

brown colonies with black centers and a black diffusible pigment. Colony morphology for other *Candida* spp. can also be detected. Bismuth and sulfide compounds are also inhibitory to many bacterial species.

Brain-heart infusion agar (fungal formulation)

Brain-heart infusion (BHI) agar, fungal formulation, with 10% sheep blood is an enrichment agar useful for the isolation of pathogenic yeast and dimorphic fungi (in yeast form) from clinical specimens. The addition of antimicrobial agents (chloramphenicol and gentamicin or penicillin and streptomycin) makes the medium selective by inhibiting the growth of bacteria. The saprophytic fungi are not inhibited by these agents. Cycloheximide can be added to inhibit the saprobes.

Canavanine-glycine–bromothymol blue agar

This differential medium distinguishes *C. neoformans* from *C. gattii*. Plates are inoculated and incubated at 30°C for 1 to 5 days. *C. gattii* (serogroups B and C) colonies appear cobalt blue, indicating the assimilation of glycine, whereas colonies of *C. neoformans* (serogroups A and D) grow poorly and leave the medium greenish-yellow. Glycine is the sole carbon source; assimilation of glycine and the subsequent conversion of creatine to ammonia creates an alkaline shift in the pH, producing blue colonies. The medium is selective for *Cryptococcus* because they are the only yeasts known to have a natural resistance to L-canavanine. Bromothymol blue is added to indicate the shift in pH to 7.0 when the media appears cobalt blue.

Chromogenic agar for *Candida*

Chromogenic agars, produced by several manufacturers, provide rapid and accurate identification of *Candida* spp. isolated in clinical laboratories. Proprietary chromogenic substrates in the agar produce different colored products based on species-specific enzymes. CHROMagar *Candida* (BD Diagnostic Systems, Sparks, MD) incorporates chloramphenicol to inhibit bacteria; this medium is more selective than Sabouraud dextrose agar. *C. albicans* colonies are a yellow-green to blue-green, *C. tropicalis* appears dark blue to metallic blue, and *C. krusei* is a light mauve to mauve-colored colony. Colonies should be examined after a 48-hour incubation under aerobic conditions at 35°C. ChromID *Candida* agar (bioMérieux, Durham, NC) is another example.

Cornmeal agar

Variations of cornmeal agar are recommended for the cultivation or enhancement of particular fungal characteristics. Cornmeal agar without dextrose (glucose) is recommended for the cultivation of chlamydospore-bearing *C. albicans*, with chlamydospore production further enhanced by the addition of 1% Tween-80. This formulation also stimulates pseudohyphae and arthroconidia production. Cornmeal agar with 1% glucose is not recommended for chlamydospore production. Rather, this formulation favors more luxuriant growth and improves pigment production that is used to differentiate *Trichophyton rubrum*, which produces a red pigment, from *Trichophyton mentagrophytes*, which does not.

Each plate should be inoculated with a known *C. albicans* control and the unknown isolate(s) as single streaks without cutting into the agar; the streak lines should be covered with a flame-warmed coverslip. The coverslip provides a reduced oxygen environment that stimulates chlamydospore formation. The plate should be incubated at room temperature in the dark and examined with a low-power microscope lens daily for up to 1 week. Isolates may become positive for chlamydospores within 48 hours but cannot be considered negative before the fifth day of culture.

Dermatophyte test medium

Dermatophyte test medium is used to isolate dermatophytes from cutaneous specimens. Chloramphenicol and gentamicin are incorporated to inhibit the growth of most bacteria, and cycloheximide inhibits saprophytic fungi. This medium turns from yellow to red because of the formation of alkaline metabolites when dermatophytes grow. Phenol red is the pH indicator. The microscopic characteristics of the dermatophyte can be seen from the colonies growing on this medium. However, pigment production cannot be evaluated because of the pH indicator.

Inhibitory mold agar

This is an enriched selective medium for the growth of cycloheximide-sensitive fungi such as *Cryptococcus*, *Histoplasma capsulatum*, and the mucoraceous fungi. Chloramphenicol and gentamicin are used to inhibit most bacteria. Nutrients are provided by casein, yeast extract, and animal tissue.

Littman oxgall agar

Littman oxgall agar is a general-purpose medium used to isolate cycloheximide-sensitive fungi, especially dermatophytes. It is similar to inhibitory mold agar in that it does not contain cycloheximide. Glucose is added as a carbon source, peptone supplies a nitrogen source, and oxgall prevents the spreading of fungal colonies so yeasts and molds will form distinct colonies and can be isolated in pure culture. Crystal violet and streptomycin are added to inhibit the growth of bacteria.

Mycosel/mycobiotic agar

Mycosel (BD Diagnostic Systems) and Mycobiotic (Thermo Fisher Scientific, Waltham, MA) are selective media used to isolate pathogenic fungi, dermatophytes, and systemic pathogens. Nutrients are provided primarily from a pancreatic digest of soybean meal and glucose. The cycloheximide suppresses the growth of saprophytic fungi, whereas the chloramphenicol inhibits bacterial contaminants.

Potassium nitrate assimilation medium, modified

Potassium nitrate (KNO_3) assimilation medium is a differential medium useful in assessing the ability of yeasts to assimilate KNO_3 . The modified medium is a slanted medium containing KNO_3 , yeast carbon base, Nobel agar, and

bromothymol blue. A single colony of the cultured isolate is picked using a sterile applicator stick, inoculated over the entire slant surface, and incubated at 25° to 30°C. The ability to assimilate potassium nitrate is indicated by a color change of the medium from greenish yellow to blue or blue green.

Potato dextrose agar

Potato dextrose agar (PDA) is used as a primary plating medium for the cultivation, enumeration, and identification of yeasts and molds from products as well as from clinical specimens. The potato infusion encourages growth of and sporulation by fungi. Dermatophyte pigment production is enhanced on this medium. It is commonly used in the slide culture technique to visualize the microscopic morphology of fungi including mycelia and reproductive structures. Tartaric is added to lower the pH to approximately 3.5, inhibiting most bacterial species.

Potato flake agar

Potato flake agar is used to induce sporulation in fungi. It is easier to prepare and more stable than potato dextrose agar. Potato flakes and dextrose provide the nutrients. To enhance the growth of fungi and slightly inhibit bacterial growth, the pH is adjusted to approximately 5.6. A modified version incorporates cycloheximide and chloramphenicol to make the medium more selective for fungi, especially dermatophytes.

Rice extract agar

Rice extract agar without additional dextrose and with Polysorbate 80 is useful in the cultivation of *C. albicans*, with enhancement of chlamydospore production. Rice extract agar with 2% dextrose has been shown to enhance pigment production by *T. rubrum*, facilitating its differentiation from *T. mentagrophytes*.

The medium should be inoculated very lightly on the surface of the agar in a manner similar to that described for inoculation of cornmeal agar. The streaks should be covered with a flame-warmed coverslip to stimulate chlamydospore production. All cultures should be incubated at 25° to 30°C for 48 hours and then up to 10 days before regarding as negative.

Rice grains medium

Rice grains medium is useful in the differentiation of *Microsporum audouinii* from other dermatophytes, especially *Microsporum canis*. *M. audouinii* grows poorly on this medium and discolors the medium. Other dermatophytes and most other fungi grow well and sporulate on this medium, with no discoloration of the medium. Sterile rice grains should be spot-inoculated to prevent confusion when differentiating between discoloration and actual growth.

Sabouraud–brain-heart infusion agar

Sabouraud–brain-heart infusion agar (SABHIA) is a general-purpose medium used to isolate fungi. It is made from a combination of Sabouraud dextrose agar and brain-heart infusion agar. This medium is particularly useful for the isolation

of dimorphic fungi from clinical specimens. The addition of blood increases the isolation of dimorphic fungi and promotes the conversion to the yeast stage. Antimicrobials such as chloramphenicol, cycloheximide, streptomycin, and penicillin can be added to increase the selectivity of the medium for fungi.

Sabouraud dextrose agar or broth (Emmons modification)

Sabouraud dextrose agar and Sabouraud dextrose broth are nutrient media suitable for the cultivation of fungi, especially those associated with cutaneous and mucocutaneous infections. The formulations are identical, with the exception of the agar. To make solid plated medium, 1.5% to 2% agar is added to the broth formulation.

The Emmons-modified Sabouraud dextrose agar is the formulation commonly used today. It has 2% dextrose and a neutral pH as opposed to 4% dextrose and an acidic pH (5.6), which were in the original formulation developed by Sabouraud. Antimicrobials can be added to make this medium more selective for fungi.

Trichophyton agars 1 to 7

This set of seven media is used for the identification of *Trichophyton* species. The nutrients vary in the seven media. Based on a growth rating of 1 to 4 for each of the seven media, the fungus is identified.

Fungal mounting fluids

KOH-glycerin

Potassium hydroxide (KOH) or sodium hydroxide (NaOH) and glycerin solution are used as the mounting fluid in the preparation of wet mounts to visualize fungi in clinical material. The 10% or 20% KOH aids in clearing the specimen of nonfungal materials, and the glycerol retards the dissolution of the fungal elements. Nails may require a greater KOH concentration (25%). After the addition of the mounting fluid, the slide is set at room temperature for 5 to 30 minutes to allow digestion to occur. Historically, heating the slide was performed to speed up the clearing process of the KOH, but overheating has the potential of producing KOH crystals or damaging fungal elements. A modification of this procedure involves combining the KOH with 40% dimethyl sulfoxide (DMSO) rather than glycerol. The DMSO facilitates the penetration of KOH into the specimen materials and speeds the clearing process without the need for heating.

Lactophenol cotton blue

Lactophenol cotton (aniline) blue is a mounting medium useful for examining the microscopic morphology of an isolated fungus. It aids in staining hyphal elements (aniline blue) and preserving fungal materials (lactic acid), and it enhances visualization by staining all chitin-containing structures a light blue. A small drop of the mounting medium is placed onto a

slide; the fungal material is added directly and teased apart or placed on the surface of cellophane tape, topped with a coverslip, and examined. Some manufacturers add 10% polyvinyl alcohol as a fixative so permanent smears can be prepared.

Fungal stains

Ascospore stain

When grown on appropriate media, ascomycetous fungi can produce ascospores. A differential stain consisting of malachite green and safranin aids in visualizing ascospores, which stain green while the vegetative portion of the fungus stains red. A portion of the fungal growth is smeared onto a glass slide and heat-fixed. The slide is flooded with 5% malachite green for 3 minutes. The slide is washed with tap water and then decolorized for 30 seconds with 95% ethyl alcohol. The counterstain, 5% safranin, is added for 30 seconds. After air-drying, the slides are examined at 400× and 1,000× magnification.

Calcofluor white stain

The use of calcofluor white stain with 10% KOH enhances the visualization of fungi in clinical specimens of skin, hair, and nails. Fungal elements, including *Pneumocystis jirovecii*, take up the fluorescent dye because it binds to cellulose and chitin, which are found only in fungal cell walls and, depending on the combination of filters used, appear brilliant green-yellow or blue-white.

For examination of clinical samples, one drop of calcofluor white solution and one drop of 10% KOH are added to the specimen on a microscope slide. A coverslip is placed on top of the mixture, and the slide should sit for 5 to 10 minutes. Then the preparation is examined using a fluorescence microscope. The microscope must have a K532 excitation filter–BG 12 barrier filter or a G-35 excitation filter–LP420 barrier filter combination.

India ink

The India ink method is useful for demonstrating the presence of a capsule. It has been used for the demonstration of *C. neoformans* in clinical specimens, particularly cerebrospinal fluid. In this method, the capsule displaces the colloidal carbon particles in the ink; thus the capsule appears as a clear halo around the yeast cell. Because of the low sensitivity of the India ink method in detecting encapsulated yeast, antigen detection methods (e.g., latex agglutination, enzyme immunoassay) are recommended. However, some of these tests are prone to false-positive results.

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Selected procedures

Donald C. Lehman and Connie R. Mahon

PROCEDURE 1

Acid-fast staining of mycobacteria

Principles

The primary stain binds to mycolic acid in the cell wall of mycobacteria, and similar organisms, and is retained after the decolorizing step with acid alcohol. The counterstain does not penetrate the mycobacteria to affect the color of the primary stain.

Application

The direct smear examination is a valuable diagnostic procedure for the detection of acid-fast organisms in clinical specimens.

Materials

TB auramine-rhodamine T stain
Carbolfuchsin stain
0.5% acid alcohol (0.5% HCl in 70% ethanol)
2% acid alcohol (prepared by laboratory)
1% sulfuric acid (partial acid-fast procedure)
0.5% aqueous potassium permanganate solution
0.3% aqueous methylene blue solution (prepared by laboratory)
Microscope slides 1 × 3 inches
Tap water

Procedures

Fluorescent stain

1. Cover smears with TB auramine-rhodamine T stain, and stain for 25 minutes.
2. Wash in running tap water.
3. Move slides to slide rack on acid alcohol collection container.
4. Flood smears with 0.5% acid alcohol, and decolorize for 2 minutes.
5. Wash smears in running tap water.
6. Move slides to original staining rack.
7. Flood smears with potassium permanganate counterstain for 4 minutes.
8. Wash smears in running tap water.
9. Air-dry.
10. Examine smears with the ×16 and ×40 objective lenses of a fluorescent microscope equipped with a filter system comparable to a BG-12 exciter filter and an OG-1 barrier filter. Examine each smear for 3 to 5 minutes.

Kinyoun stain

1. Cover smears with carbolfuchsin, and stain for 5 minutes.
2. Wash slides with running tap water.
3. Move slides to staining rack on acid alcohol collection container.
4. Decolorize with acid alcohol until no more color appears in the washings.
5. Wash slides with running tap water and move them to original staining rack.
6. Flood slides with methylene blue counterstain for 1 minute.

7. Wash with running tap water, drain, and air-dry.
8. Examine smears with ×100 oil immersion lens.

Modified Kinyoun stain (partial acid-fast)

1. Flood the slides with carbolfuchsin stain for 5 minutes.
2. Rinse with running tap water.
3. Flood slides with 70% ethanol, and rinse with tap water. Repeat until excess red dye is removed.
4. Move slides to rack on an acid collection container.
5. Continuously drop 1% sulfuric acid on the smear until the washing becomes colorless.
6. Rinse with running tap water.
7. Move slides to original staining rack.
8. Counterstain with methylene blue for 30 seconds.
9. Rinse with running tap water, and air-dry.
10. Examine smears with ×100 oil immersion objective lens.

Ziehl-Neelsen stain

1. Cover smear with a piece of filter paper cut slightly smaller than the slide.
2. Layer filter paper with carbolfuchsin stain. With a Bunsen burner, heat the smears gently until steaming occurs. Stain for 5 minutes without additional heating.
3. Proceed as for Kinyoun method, beginning with Step 2.

Results

Fluorescent stain

Mycobacteria stain bright orange. Count the number of acid-fast bacilli seen on the smear, and report as follows:

Number of acid-fast bacilli/oil immersion field (OIF)	Report
1–20	Number seen
21–80	Few
81–300	Moderate
>300	Numerous

Kinyoun and Ziehl-Neelsen stains

Mycobacteria stain red, whereas the background material and non-acid-fast bacteria stain blue. *Nocardia* spp. will weakly stain red in the modified Kinyoun stain. The coccidian species (*Cryptosporidium*, *Cystoisospora*, and *Cyclospora*) will also stain red.

Controls

Positive: *Mycobacterium tuberculosis*
Negative: *Bacillus subtilis*

PROCEDURE 2

Bacitracin susceptibility

Purpose

To differentiate *Streptococcus pyogenes* from other β -hemolytic streptococci

Principle

Group A streptococci are susceptible to low levels (0.04 units) of bacitracin, whereas other groups of β -hemolytic streptococci are resistant. Rare strains of group A streptococci are resistant (approximately 1%), whereas some strains of groups B, C, and G streptococci are susceptible (5% to 10%). Susceptibility to bacitracin presumptively identifies an isolate as *S. pyogenes*. This procedure was designed for use only with pure cultures; however, some clinical microbiologists will add a bacitracin disk to a primary throat culture to screen for *S. pyogenes*.

Specimen

Isolated colonies of test organism on sheep blood agar

Media

5% sheep blood agar plate

Reagent

Bacitracin disk, 0.04 units

Procedure

1. Streak surface of agar plate to obtain isolated colonies.
2. Aseptically place bacitracin disk onto inoculated surface. Press down gently on disk to ensure complete contact with agar surface.
3. Incubate plate at 35° C for 18 to 24 hours in a CO₂ incubator.

Interpretation

Susceptible: Any zone of inhibition around the bacitracin disk
Resistant: Uniform lawn of growth up to the edge of the disk (see Fig. 15.16)

Controls

Positive (susceptible): *Streptococcus pyogenes*
Negative (resistant): *Streptococcus agalactiae*

PROCEDURE 3

Bile esculin hydrolysis

Purpose

To differentiate group D streptococci and enterococci from other catalase-negative, gram-positive cocci

Principle

Group D streptococci and enterococci grow in the presence of bile, metabolize glucose, and also hydrolyze esculin to esculetin. Esculetin diffuses into the agar and combines with ferric citrate in the medium to produce a black complex.

Specimen

Isolated colonies of test organism on sheep blood agar

Media

Bile esculin agar

Procedure

1. Pick one or two isolated colonies from a sheep blood agar plate, and inoculate to bile esculin agar medium. A single plate can be divided into several pie-shaped sections for inoculation of multiple test organisms.

2. Incubate plate or slant at 35° C for 18 to 24 hours. A positive result is often seen within 4 hours. A negative result should be incubated for an additional 24-hour period.

Interpretation

Positive result: Blackening of the agar
Negative result: No blackening of the agar

Note: Growth alone does not constitute a positive result.

Controls

Positive: Group D streptococci
Negative: *Streptococcus pyogenes* or viridans streptococci

PROCEDURE 4

Bile solubility

Purpose

To differentiate *Streptococcus pneumoniae* from other α -hemolytic streptococci.

Principle

The bile (sodium deoxycholate) solubility test distinguishes *S. pneumoniae* from all other α -hemolytic streptococci. *S. pneumoniae* is bile-soluble, whereas all other α -hemolytic streptococci are bile-resistant. Sodium deoxycholate (2% in water) will lyse the pneumococcal cell wall. Bile salts, specifically sodium deoxycholate and sodium taurocholate, have the capability to lyse *S. pneumoniae* selectively when added to actively growing bacteria in agar or broth media.

Reagents

Sodium deoxycholate (10%)

Procedure

Plate spot test

1. Place 1 drop of 10% bile solubility reagent near suspected 18- to 24-hour-old colonies growing on sheep blood agar. Gently

rotate the agar plate, rolling the drop over several representative colonies. Take care not to dislodge the colonies.

2. Incubate the plate aerobically in an upright position at 35° C, and examine periodically for up to 30 minutes. Leave lid slightly ajar to enhance evaporation of the reagent.
3. Observe the colony for disintegration or solubility.

Interpretation

Positive result: Disintegration of colonies and/or the appearance of an α -hemolytic zone on the plate where the colony was located within 30 minutes.

Negative result: Colonies on the plate remain intact with no change in colony integrity within 30 minutes.

Controls

Positive: *Streptococcus pneumoniae*

Negative: α -Hemolytic or viridans streptococci

PROCEDURE 5

Calcofluor white stain

Principle

Calcofluor white (see [Appendix C](#)) is a colorless dye that binds to cellulose and chitin found in fungi. It fluoresces when exposed to long-wavelength ultraviolet and short-wavelength visible light. Special filters are required for optimal use. Evans blue is used as a counterstain and decreases fluorescence of host tissues and cells. Potassium hydroxide (KOH) can be added to clear the specimen of host material.

Application

Calcofluor white may be used as a specific stain for rapid screening of clinical specimens for fungal elements. This stain may be useful when morphology is ambiguous and the nonspecific staining of other techniques such as Gomori methenamine silver stain gives indeterminate results.

Materials

Stock solution

A 1% (wt/vol) aqueous solution of calcofluor white is prepared by dissolving the powder in distilled water with gentle heating. The stock solution is stable for 1 year at room temperature.

Working solution

0.1% calcofluor white M2R containing 0.01% to 0.08% Evans blue

Procedure

1. Place the specimen onto a glass microscope slide.
2. Add 1 to 2 drops of working calcofluor white solution. 1 drop of 10% KOH can be added to clinical specimens. If KOH is added, the slide can be gently heated to enhance tissue digestion.
3. Coverslip and let the specimen sit for 1 to 2 minutes.
4. Cover the slide with a paper towel, and gently press to remove any excess fluid.
5. Examine specimen on a fluorescent microscope, under $\times 10$ and $\times 40$ objective lenses using the following set of filters: G 365, LP 450, and FT 395.

Results

Yeast cells, pseudohyphae, and hyphae display a bright apple-green or blue-white fluorescence, depending on which barrier filter is used. *Pneumocystis* cysts, 5 to 8 μm , also fluoresce and exhibit a characteristic peripheral cyst wall staining with an intense internal "double-parenthesis-like" structure.

PROCEDURE 6

Calibration of the ocular micrometer

Purpose

To calibrate an ocular micrometer to determine the size of parasites and formed elements examined microscopically

Principle

Size is an important criterion in the identification of parasites, especially those found in clinical specimens from the digestive tract. Calibrating an ocular micrometer allows laboratory scientists to determine the size of objects seen during a microscopic examination of clinical material.

Procedure

1. Insert the ocular micrometer into the eyepiece of the microscope so the zero of the scale is on the left side.
2. Place the calibrated stage micrometer on the stage, and focus on the scale using the low-power ($\times 10$ objective) lens. The stage micrometer is divided into major lines separated by 0.1 mm ($100\mu\text{m}$) and minor lines separated by 0.01 mm ($10\mu\text{m}$).
3. While using low power, align the zero on the left side of the stage micrometer with the zero on the left side of the ocular micrometer. Do not move the stage micrometer after this step.

4. Scan the two scales for a point where a division line on the ocular micrometer directly aligns with a division line (minor line) on the stage micrometer.
5. Count the number of stage units and ocular units at this point. Divide the number of stage units by the number of ocular units, and multiply the result by 10. This gives the value (in micrometers) for 1 ocular unit on low power.

$$\text{Micrometers/ocular unit} = \left(\frac{\text{No. of stage units}}{\text{No. of ocular units}} \right) \times \left(\frac{10\mu\text{m}}{\text{Stage unit}} \right)$$

6. Repeat the procedure at high power ($\times 40$ objective lens) and with the $\times 100$ objective lens to get the value of one ocular unit at each of these magnifications. To calculate the size of an organism, count the number of ocular units, multiply by the value for an ocular unit at that magnification, and report the value in micrometers.

PROCEDURE 7

CAMP test

Purpose

To differentiate *Streptococcus agalactiae* (group B *Streptococcus*) from other β -hemolytic streptococci

Principle

S. agalactiae produces CAMP (named after Christie, Adkins, Munch-Peterson) factor, which enhances the lysis of sheep red blood cells by staphylococcal β -lysin. A positive reaction can be observed in 5 to 6 hours with incubation in CO_2 (18 hours with incubation in ambient air).

Specimen

1. Isolated colonies of test organism on sheep blood agar
2. β -Lysin-producing *Staphylococcus aureus* on sheep blood agar

Medium

Sheep blood agar plate

Procedure

1. Inoculate *S. aureus* along a straight line down the center of the agar plate.

2. Inoculate the streptococcal isolates along a thin line about 2 cm long and perpendicular to, but not touching, the *S. aureus* streak.
3. Incubate plate at 35°C for 18 hours.

Interpretation

Positive result: Arrowhead-shaped area of enhanced hemolysis where the two streaks (staphylococcal and streptococcal) approach each other (see Fig. 15.17)

Negative result: No enhanced hemolysis

CAMP inhibition reaction (reverse CAMP-positive): Inhibition of hemolysis by *S. aureus* where the two streaks approach each other

This reaction is characteristic of *Arcanobacterium haemolyticum* caused by a phospholipase D.

Controls

Positive: *Streptococcus agalactiae*

Negative: *Streptococcus pyogenes*

PROCEDURE 8**Catalase test****Purpose**

To differentiate organisms that produce the enzyme catalase from those that do not produce catalase

Principle

Staphylococci produce an enzyme, catalase, which reacts with peroxide to liberate oxygen. When a colony is emulsified in 3% hydrogen peroxide, the resultant bubbles on the slide can be visualized, which constitutes a positive test result.

Reagent

3% Hydrogen peroxide

Procedure

1. Place 1 drop of hydrogen peroxide on a clean glass slide.

2. Pick the colony in question from an agar plate with a wooden applicator stick.
3. Touch the stick containing the bacteria to the drop of hydrogen peroxide, and observe for immediate bubbling. The reaction is positive if there is a rapid evolution of gas.

Note: The enzyme catalase is present in red blood cells. Colonies to be tested should not be taken directly from a blood agar plate.

Interpretation

Positive result: Bubbling

Negative result: No bubbling

Controls

Positive: *Staphylococcus aureus*

Negative: *Streptococcus pyogenes*

PROCEDURE 9**Coagulase test (tube)****Purpose**

To differentiate and identify staphylococcal species that produce the enzyme coagulase

Principle

The tube coagulase test detects the presence of free coagulase enzyme produced by *S. aureus*. This extracellular enzyme forms a clot when a bacterial suspension is incubated with plasma. The screening test by the slide method detects only cell-bound coagulase; therefore any negative slide coagulase test must be confirmed with the tube coagulase test.

Specimen

A broth culture of the organisms to be tested or a single colony from a blood agar plate

Reagent

Coagulase plasma (BD BBL; Becton Dickinson, Franklin Lakes, NJ)

Procedure

1. To 0.5 mL of undiluted rabbit plasma, add 1 loopful of growth from 18- to 24- hour agar culture, 0.1 mL of broth culture, or a single colony from a blood agar plate.
2. Incubate in a heating block at 35° C, and examine for clotting at 30-minute intervals for 4 hours.

3. If no clot is observed at the end of 4 hours, let stand at room temperature for 18 to 24 hours. Observe for clotting.

Interpretation

A positive coagulase test result is represented by any degree of clotting from a loose clot suspended in plasma to a solid clot. The majority of coagulase-positive strains produce a clot within 4 hours.

Notes

1. A false-positive test result may occur with mixed cultures or with a pure culture of a gram-negative bacillus, but the clotting mechanism is different. Therefore organisms to be tested must first be determined to possess characteristics consistent with the genus *Staphylococcus*.
2. *S. aureus* also produces the fibrinolysin enzyme staphylokinase, which lyses the clot. It is important to examine for a clot after 4 hours of incubation, or a false-negative result can occur.

Controls

Positive: *Staphylococcus aureus*

Negative: *Staphylococcus epidermidis*

PROCEDURE 10

Corn Meal Agar for yeast identification

Purpose

To aid in the identification of pathogenic yeasts by microscopic morphology

Principle

Isolated yeasts are inoculated onto the surface of a Corn Meal agar with Polysorbate (tween) 80 plate (see [Appendix C](#)). The inoculum is covered with a coverslip and incubated for 3 days. The yeasts on the plate are examined microscopically. Microscopic morphology is an important characteristic in the identification of yeasts.

Specimen

Isolated cultures of yeasts

Medium

Corn Meal Agar with Tween 80 plate; one third or one fourth of a plate can be used for each yeast.

Procedure

1. Pick up a small amount of a yeast colony with an inoculating loop.

2. Make one streak of the yeast in the center of the agar surface. Do not cut the agar.
3. Make three or four streaks across the original streak to dilute the inoculum, being careful not to cut into the agar. Stab the medium twice through a streak line being careful not to touch the bottom of the Petri dish.
4. Cover the inoculum with a sterile coverslip, and incubate at room temperature in the dark for 2 days. If the results are negative, reincubate an additional 48 to 72 hours.
5. Remove the lid from the Petri dish, and examine the yeast with the low-power (×10) and high-power (×40) objective lenses for the presence of hyphae, pseudohyphae, arthroconidia, chlamydoconidia, and blastoconidia.

Controls

Candida albicans is used to demonstrate hyphae and chlamydospores.

Cryptococcus spp. are used to demonstrate blastoconidia.

PROCEDURE 11

Germ tube production for yeast identification

Purpose

To differentiate among the pathogenic yeast based on the ability to produce a germ tube

Principle

When grown in serum or plasma at 35° C, some yeasts form hyphae. This is an important characteristic in the identification of *Candida albicans*.

Specimen

Isolated cultures of yeasts

Medium

Rabbit plasma or serum or fecal calf serum

Procedure

1. Make a light suspension by adding one yeast colony to 0.5 mL of sterile serum. Remel Germ Tube Solution (Thermo Fisher Scientific, Waltham, MA), composed of fetal bovine serum and

trypticase soy broth, may be used as an alternative. This alternative substrate eliminates the risk of human immunodeficiency virus and hepatitis viruses that can be present in human serum.

2. Incubate the suspension at 35° C for 2.5 to 3 hours.
3. Place 1 drop of the suspension on a glass microscope slide, and add a coverslip.
4. Observe microscopically for the presence of germ tubes. Hyphae budding from a yeast cell without a constriction is a true germ tube. If there is a constriction at the point of budding, that is a pseudogerm tube.

Controls

A known germ tube positive isolate of *C. albicans* can serve as the positive control; *Cryptococcus* spp. can be used as a negative control.

PROCEDURE 12

Gram stain

Principles

The Gram staining method was developed by the Danish bacteriologist Christian Gram in 1884. The sequential steps provide for crystal violet (hexamethyl-*p*-rosaniline chloride) to color all cells and background material a deep blue and for Gram's iodine to provide the larger iodine element to replace the smaller chloride in the stain molecule. Bacteria with thick cell walls containing teichoic acid retain the crystal violet-iodine complex dye after decolorization and appear deep blue; they are *gram-positive bacteria*. Other bacteria with thinner walls containing lipopolysaccharides do not retain the dye complex; they are *gram-negative bacteria*. The alcohol-acetone decolorizer damages these thin lipid walls and allows the stain complex to wash out. All unstained elements are subsequently counterstained red by safranin dye. The differential ability of the Gram stain makes it useful in microbial taxonomy.

Application

The Gram stain is used routinely in the clinical microbiology laboratory for the primary microscopic examination of specimens submitted for smear and culture. It is ideally suited for specimen types in which bacterial infections are strongly suspected, but it may be used to characterize any specimen. Cerebrospinal fluid, sterile fluids, expectorated sputum or bronchoalveolar lavages, and wounds and exudates are routinely stained directly. The Gram stain is regularly used to characterize bacteria growing on culture media.

Materials

Crystal violet
Gram's iodine solution
Decolorizer (95% ethanol or 1 : 1 ratio of acetone and 95% ethanol)
Safranin
Microscope slides 1 × 3 inches
Tap water

Procedure

1. Dry the material on the slide so it does not wash off during the staining procedure. Adherence can be improved by fixation in 70% to 95% alcohol or by gently warming the slide (heat fixation) to remove all water from the material.
2. Place the smear on a staining rack, and overlay the surface of the material to be stained with the stains in sequence as shown in the figures.
3. Place the smear in an upright position in a staining rack, allowing the excess water to drain off and the smear to dry. Never blot a critical smear (e.g., cerebrospinal fluid). Never put immersion oil on a smear until it is completely dry.
4. Examine the stained smear using the low-power objective lens, and select an area to examine more closely using a ×40 to ×100 oil objective lens. Suspicious areas are evaluated using the ×100 oil objective lens of the microscope.

Results

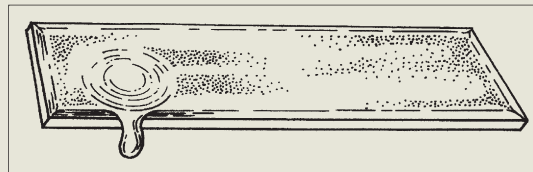
Gram-positive bacteria stain dark blue to blue-black. All other elements stain red with safranin. Individual structures absorb a different amount of safranin, so some have prominent staining (strong avidity), and others are weakly stained (low avidity). Among the gram-negative bacteria, the enterics have strong avidity and stain bright red; pseudomonads are less avid and stain moderately well. Anaerobic bacilli and other thin-walled gram-negative organisms, such as *Borrelia*, *Legionella*, and *Spirillum* spp., stain weakly. Always check the quality of the stain and the results of the quality control organisms before interpretation. Basic fuchsin (0.1%) is sometimes used as a counterstain to enhance visualization of weakly staining gram-negative bacteria.

Precautions

The Gram stain reaction may vary from the expected in numerous well-recognized circumstances. If the crystal violet is rinsed too vigorously before it is complexed with the iodine, it will wash away and leave poor or no staining of gram-positive organisms. If the decolorization is too vigorous or prolonged, the gram-positive complex will be removed, and the normally gram-positive organisms will not stain blue. If the decolorization is insufficient, organisms may be falsely

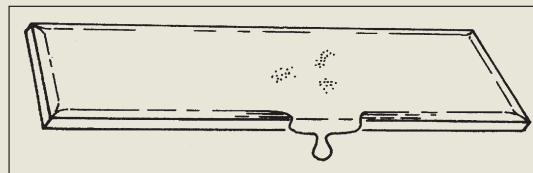
interpreted as gram-positive, and organisms in the thicker areas of the sample may be obscured. If the safranin is left on the slide for a prolonged period (minutes), the gram-positive complex will be leached from the positive cells; however, failure to leave the safranin in place for sufficient time will result in failure to stain gram-negative bacteria and background materials. Gram stain characteristics may be atypical in antimicrobial-treated and dead or degenerating organisms. Typical morphotypes should be sought. Any sample that raises questions about the quality of the stain or the method should be restained.

The crystal violet stain is not bound into the organism until the iodine is added. Any rinsing between the crystal violet and the iodine steps must be very brief.

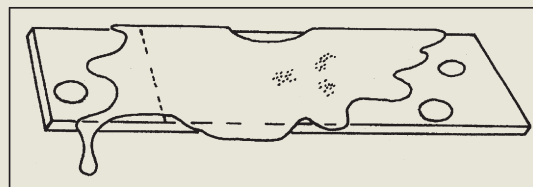


Flood the slide with crystal violet, and allow it to stand for 30 seconds.

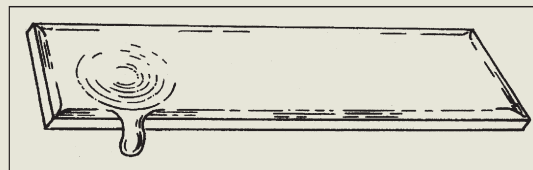
The dilute iodine solution can be used to wash away the crystal violet, and no water rinse is employed.



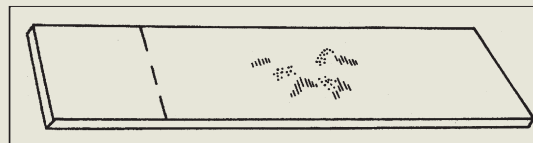
Flood with Gram's iodine, and allow it to stand for 30 to 60 seconds.



Decolorize the slide with a 1 : 1 mixture of the two decolorizers, acetone and 95% ethanol, and wash immediately with water. Acetone is a more rapid decolorizer and may give better results, but the reaction must be stopped with water as soon as the purple color disappears.



Flood with safranin or 0.1% basic fuchsin for 30 seconds to 1 minute (anaerobes). The counterstain must not be left in place for too long. Anaerobes may stain better at 1 minute or with carbol-fuchsin. Rinse very lightly with water.



Check the staining reactions of quality control organisms before proceeding with smear interpretation.

Controls

Gram positive: *Staphylococcus aureus*
Gram negative: *Escherichia coli*

PROCEDURE 13

Hippurate hydrolysis test

Purpose

To differentiate *Streptococcus agalactiae* (group B *Streptococcus*) from other β -hemolytic streptococci

Principle

The enzyme hippuricase hydrolyzes hippuric acid to form sodium benzoate and glycine. Subsequent addition of ninhydrin results in the release of ammonia from the oxidative deamination of the α amino group in glycine as well as the reduced form of ninhydrin, hydrindantin. The ammonia reacts with residual ninhydrin and hydrindantin to produce a purple-colored complex. Some isolates of group D streptococci also hydrolyze hippurate; however, these isolates are less likely to be β -hemolytic; therefore their colony morphology is different from that of group B *Streptococcus*. An isolate that is hippurate positive and bile esculin negative has a very high probability of being *S. agalactiae*.

Specimen

Isolated colonies of test organism on sheep blood agar

Reagents

Sodium hippurate, 1%

Sodium hippurate: 1 g

Distilled water: 100 mL

Dispense 0.5-mL aliquots into small capped vials. Store at -20°C . Storage life is 6 months.

Ninhydrin reagent

Ninhydrin: 3.5 g

Acetone-butanol mixture (1 : 1): 100 mL

Store at room temperature. Storage life is 12 months.

Procedure

1. Inoculate the solution of sodium hippurate heavily with colonies 18 to 24 hours old until a milky suspension is obtained.
2. Incubate tube for 2 hours at 35°C .
3. Add 0.2 mL of ninhydrin reagent.
4. Mix and incubate for 10 to 15 minutes at 35°C .

Interpretation

Positive result: Deep purple color—indicates hippurate hydrolysis

Negative result: No color change or very slight purple color

Controls

Positive: *Streptococcus agalactiae*

Negative: *Streptococcus pyogenes*

PROCEDURE 14

Leucine aminopeptidase

Purpose

To differentiate and identify catalase-negative, gram-positive cocci such as *Aerococcus* and *Leuconostoc* from *Streptococcus*, *Enterococcus*, *Lactococcus*, and *Pediococcus*.

Principle

Based on the ability of catalase-negative, gram-positive cocci to hydrolyze leucine- β -naphthylamide by the enzyme leucine aminopeptidase (LAP) produced by LAP-positive organisms. The enzymatic activity releases β -naphthylamine, which when p-dimethylaminocinnamaldehyde reagent is added forms a highly visible red color.

Reagents

Remel LAP Disc w/Reagent (Thermo Fisher Scientific)

Procedure

1. Aseptically place a LAP disk in a sterile petri dish, and allow it to warm to room temperature.

2. Slightly dampen the LAP disk with reagent grade water or with a small amount of sterile distilled water.
3. Using a wooden applicator stick, rub a small amount of several colonies of an 18-to-24-hour pure culture onto a small area of the LAP disk.
4. Incubate at room temperature for 5 minutes.
5. After the incubation period, add 1 drop of cinnamaldehyde reagent, and read within 1 minute.

Interpretation

Positive result: Development of a red/pink color

Negative result: No change or a slight yellow color

Controls

Positive: *Enterococcus faecalis*

Negative: *Aerococcus viridans*

PROCEDURE 15

NALC-sodium hydroxide digestion-decontamination

Purpose

To enhance the recovery of mycobacteria from clinical specimens by reducing the number of contaminating or commensal bacteria

Principle

Sodium hydroxide (NaOH) acts as both a decontaminating agent and digestant. Because of the toxicity of NaOH to mycobacteria, the agent should be used at the lowest concentration that inhibits the growth of contaminating bacteria. *N*-acetyl-L-cysteine (NALC) is a mucolytic agent that allows for a lower concentration of NaOH to be used and thereby optimizes the recovery of mycobacteria from the specimen. Decontamination should only be done on specimens likely to be contaminated with normal microbiota, such as sputa.

Reagents

NALC-NaOH digestant: Combine equal volumes of 2.94% sodium citrate dihydrate (0.1 M) and 4% NaOH. Just before use, add 0.5 g of powdered NALC/100 mL of mixture. Refrigerate when not in use; discard after 24 hours.

Phosphate buffer (0.067 M, pH 6.8) or sterile, distilled water, 30 to 40 mL per specimen

Bovine albumin fraction V, 0.2% in sterile saline

Procedure

1. Working in a biological safety cabinet, transfer 10 mL of the specimen (or total specimen, if volume is less than 10 mL) to a 50-mL, screw-capped centrifuge tube. If the specimen volume is greater than 10 mL, select the most purulent-appearing material. Add an equal volume of NALC-NaOH digestant to each sample.
2. Tighten caps and mix on a vortexer until liquefied (5 to 20 seconds), inverting each tube to ensure that the NALC-NaOH

solution contacts any untreated particles on the upper part of the tube.

3. Let the tubes stand at room temperature for 15 minutes. If more decontamination is desired, increase the concentration of NaOH rather than the time the specimen is exposed to the digestion-decontamination mixture.
4. Dilute the digested-decontaminated specimens to the 50-mL mark with sterile distilled water or sterile phosphate buffer to minimize the continuing action of the NaOH and lower the specific gravity of the specimen. Tighten caps, and invert or swirl to mix.
5. Centrifuge at $\times 3000g$ for 15 minutes (or the appropriate combination of relative centrifugal force and time to give 95% sedimentation) using aerosol-free safety cups or in an aerosol-controlled vented refrigerated centrifuge.
6. Holding the tube so the sediment is on the upper side of the tube, pour off the supernatant into a splashproof discard container of disinfectant.
7. Holding the tube in a horizontal position to keep the sediment as dry as possible, use a sterile applicator stick to remove a small part of the sediment, and place it on a marked microscope slide. The smear should be about 1×2 cm.
8. Resuspend sediment in 1 to 2 mL of sterile 0.2% bovine albumin solution. If the medium will be inoculated immediately, the sediment may be resuspended in sterile water or sterile saline.
9. An optional step is to prepare a 1:10 dilution using 0.5 mL of the resuspended sediment in 4.5 mL of sterile water. Dilution decreases the concentration of toxic substances that may inhibit the growth of mycobacteria. Inoculate diluted and undiluted specimens to solid media.

PROCEDURE 16

Novobiocin susceptibility test

Purpose

To differentiate *Staphylococcus saprophyticus* from other coagulase-negative staphylococci (CoNS)

Principle

S. saprophyticus is a significant isolate in urine samples of women. *S. saprophyticus* must be differentiated from other CoNS such as *S. epidermidis*. *S. saprophyticus* is resistant to a 5- μ g novobiocin disk, whereas other CoNS are susceptible.

Note: Other CoNS that are resistant to 5- μ g novobiocin are *S. xylosus*, *S. cohnii*, and *S. sciuri*, but they are uncommon isolates from the urine.

Reagents

BBL Taxo Differentiation-Disc Novobiocin 5- μ g (BD Diagnostics, Sparks, MD)

Procedure

1. Prepare a suspension of the unknown *Staphylococcus* sp. in saline equal to a McFarland 0.5 standard.

2. Using a swab, inoculate the entire surface of a Mueller-Hinton plate in three directions. Allow the inoculum to dry with lid in place for 5 minutes.
3. Place a 5- μ g novobiocin disk at the center of the inoculum. Repeat the procedure using the positive and negative controls.
4. Incubate the cultures at 35° C in an ambient incubator for 18 to 24 hours.
5. After 18 to 24 hours of incubation, determine if the test organism is susceptible (≥ 16 mm) or resistant (< 16 mm).

Interpretation

≥ 16 mm = susceptible

< 16 mm = resistant

Controls

Susceptible: *Staphylococcus epidermidis*

Resistant: *Staphylococcus saprophyticus*

PROCEDURE 17

Optochin susceptibility test

Purpose

To differentiate *Streptococcus pneumoniae* from other α -hemolytic streptococci

Principle

The optochin susceptibility test is based on the differential susceptibility of *S. pneumoniae* and other hemolytic streptococci to ethyl hydroxycupreine hydrochloride. *S. pneumoniae* is inhibited by less than 5 mg/mL of this agent. When contained within a paper disk and applied to an agar surface, as for the disk diffusion antimicrobial susceptibility test, the inhibitory zones obtained with *S. pneumoniae* significantly exceed those obtained with other α -hemolytic streptococci.

Reagents

The Optochin Disc and the BBL Taxo P Disc (BD Diagnostics) are impregnated with ethyl hydroxycupreine hydrochloride (5 μ g per disk).

Procedure

1. Streak suspicious colonies on a sheep blood agar plate covering an area 3 cm in diameter.
2. Place an optochin disk in the center of the streaked area, pressing gently with sterile forceps.
3. Invert the plate, and incubate at 35° for 18 to 24 hours. After incubation, measure the zone of inhibition around the disk.

Interpretation

Zone diameter of inhibition >15 mm, *S. pneumoniae*
Zone diameter of inhibition <15 mm, α -hemolytic *Streptococcus viridans* group

Controls

Positive: *Streptococcus pneumoniae*
Negative: α -Hemolytic *Streptococcus viridans* group

PROCEDURE 18

Oxidase test

Purpose

To identify organisms that produce the enzyme cytochrome oxidase

Principle

Neisseria spp. and some gram-negative rods produce indophenoloxidase, which becomes pink to dark red and finally black when colonies contact the oxidase reagent.

Specimen

Isolated bacterial colonies growing on sheep blood or chocolate agar plates

Reagents

Oxidase reagent, 1% aqueous solution of N,N,N',N' tetramethyl-para-phenylenediamine dihydrochloride (TMPD). Available as a commercial, ready-to-use preparation. Cepti-Seal Oxidase Reagent (BD BBL and BD Difco), or disks

Procedure

1. Place 2 to 3 drops of reagent on a piece of Whatman no. 1 filter paper.

2. Remove a suspected colony from the plate with a sterile applicator stick, and smear onto the reagent saturated paper.
3. Observe for color changes.
4. Microorganisms are oxidase positive when the color changes to dark purple within 5 to 10 seconds.

Note: Microorganisms are delayed oxidase positive when the color changes to purple within 60 to 90 seconds. The modified oxidase test for gram-positive cocci uses 6% TMPD in dimethyl sulfoxide.

Interpretation

An oxidase-positive reaction is a purple color on the filter paper in 5 to 10 seconds.
Microorganisms are oxidase negative if the color does not change or it takes longer than 2 minutes

Controls

Positive: *Pseudomonas aeruginosa*
Negative: *Escherichia coli*

PROCEDURE 19

δ -aminolevulinic acid porphyrin test (disk method)

Purpose

To differentiate *Haemophilus* spp. that require exogenous X factor (hemin) for growth

Principle

Based on the ability of *Haemophilus* spp. to synthesize protoporphyrin intermediates in the synthesis of heme from the substrate δ -aminolevulinic acid (ALA). *Haemophilus* spp. that require exogenous X factor (hemin) for growth are not capable of synthesizing protoporphyrins from ALA, hence are negative with this test. Species that do not require exogenous X factor for growth possess the enzymes that convert the substrate ALA to protoporphyrin compounds and are therefore ALA-porphyrin test positive. The porphyrin test can be performed in agar, in broth, or on a disk.

Reagents

Commercially available ALA-impregnated filter paper disks (Thermo Fisher Scientific)

Procedure

1. Place the disk in a sterile petri dish. Moisten the disk with sterile water.
2. Using a sterile loop or applicator stick, inoculate the disk with the organism from a pure 18- to 24-hour growth culture medium.
3. Incubate in ambient air at 35° C for up to 6 hours. After 6 hours, observe the disk under an ultraviolet light (Wood's light).

Interpretation

Brick-red fluorescence in the area of inoculation indicates a positive test result. These *Haemophilus* spp. can produce hemin and therefore do not require it in culture media. No fluorescence is a negative test result.

Controls

Positive: *Haemophilus parainfluenzae*
Negative control: *Haemophilus influenza*

PROCEDURE 20**L-Pyrrolidonyl α -naphthylamide hydrolysis test****Purpose**

To differentiate gram-positive cocci that hydrolyze the substrate L-pyrrolidonyl α -naphthylamide (PYR) from those that are PYR-negative

Principle

PYR-impregnated disks serve as the substrate to produce α -naphthylamine, which is detected in the presence of D-dimethylaminocinnamaldehyde (DMCA) by the production of a red color.

Specimen

Isolated colonies of test organism on sheep blood agar

Procedure

1. Lightly moisten a PYR-impregnated disk with sterile water.

2. Using a sterile loop or wooden applicator stick, rub one or more isolated colonies onto the surface of the disk.
3. **Note:** Incubation time and temperature vary slightly by manufacturer. Incubate disk as indicated in the manufacturer's instructions, generally 2 to 15 minutes.
4. Add 1 drop of color developer, and observe for a red color on the disk within 5 minutes.

Interpretation

Positive result: Red color

Negative result: Colorless

Controls

Positive: *Enterococcus faecalis*

Negative: *Streptococcus agalactiae*

PROCEDURE 21**Salt tolerance test****Purpose**

To differentiate gram-positive cocci that grow in 6.5% NaCl from those that are inhibited by this salt concentration

Principle

Enterococcus, *Aerococcus*, and some species of *Pediococcus* and *Leuconostoc* can withstand a higher salt concentration than other gram-positive cocci.

Specimen

Isolated colonies of test organism on sheep blood agar

Medium

6.5% NaCl broth, nutrient broth base

Procedure

1. Pick one or two isolated colonies from a sheep blood agar plate and lightly inoculate 5 mL of NaCl broth.
2. Incubate broth at 35° C for 3 days. Check daily for growth.

Interpretation

Positive result: Turbidity

Negative result: No turbidity

Controls

Positive: *Enterococcus faecalis*

Negative: *Streptococcus pyogenes* or viridans streptococci

PROCEDURE 22**Slide culture for the identification of fungi****Purpose**

To aid in the identification of molds by microscopic appearance

Principle

Isolated fungi are inoculated onto a block of agar lying on a microscope slide. The agar block is covered with a coverslip and incubated until mature growth of the fungus is noted. The coverslip is removed, and the material attached to the coverslip is stained and examined microscopically. Microscopic morphology is an important characteristic in the identification of fungi, particularly molds.

Specimen

Isolated cultures of test fungi

Medium

Potato dextrose agar or similar fungal medium

Procedure

Prepare slide cultures in a biological safety cabinet. Fungi suspected of being pathogens are not recommended for observation in slide cultures.

1. Place a piece of filter paper into the bottom of a 100-mm sterile Petri dish.

2. Place a bent glass rod, two pieces of plastic tubing, or the bent end of a flexible straw on the filter paper.
3. Lay a clean, flamed, glass microscope slide on top of the bent glass rod, two pieces of plastic tubing, or the bent end of a flexible straw.
4. Cut a 1-cm $\times$ 1-cm square block of fungal medium, such as potato dextrose agar, from a Petri dish, and aseptically transfer the block to the microscope slide.
5. Inoculate the four sides of the agar block with the test fungus by using a heavy-gauge teasing needle or a sterile wooden applicator stick.
6. Cover the block with a flamed sterile coverslip.
7. Moisten the filter paper with sterile water and place the lid on the Petri dish.
8. Incubate the slide culture at room temperature (22° C). Examine the culture periodically for growth, and add more water as necessary. The Petri dish can be placed on a microscope stage and the culture examined with the $\times 10$ objective lens.
9. When conidia or spores are evident, carefully lift the coverslip off the agar with forceps, and place the coverslip into 1 drop of lactophenol cotton blue on another microscope slide for examination.

PROCEDURE 23

Thermometer calibration

Labeling

1. Create a calibration log sheet.
2. Etch a number on the back of each thermometer using a diamond-tipped marker.

Calibration method

3. Place the reference thermometer and thermometer(s) to be calibrated in an ice bath.
4. When the reference thermometer reads 0°C, take the reading of all thermometer(s) being calibrated, and record the readings on the log sheet.
5. Repeat steps 1 and 2 at all other applicable temperatures, such as 35°C and 56°C.

Calculation of correction factor

6. Correct for the difference in readings between the reference thermometer (reference temperature) and that of each thermometer being calibrated by subtracting the higher reading from the lower reading. If the thermometer being calibrated reads less than the reference thermometer, assign a plus (+) sign to the result of the subtraction step. This value must be added to the thermometer reading to equal the reading that would be taken from the reference thermometer. Conversely, if the reading of the

thermometer being calibrated is greater than the reference thermometer, assign a minus (–) sign to the result. This value must then be subtracted from that thermometer's reading to equal the reference value.

7. Correct for the difference between the reading of the reference thermometer and the "real" temperature using the process just described. Add the correction factor for the reference thermometer to the correction factor calculated in step 1 for each thermometer being calibrated.
8. Determine the correction factor at each temperature for each thermometer, and record it on the log sheet for the thermometer. The log sheet should be kept for the life of the thermometer.
9. Tolerance limits are set by each laboratory but are usually $\pm 1^\circ\text{C}$. Discard all thermometers that exceed the established tolerance limit.

Examples of correction factors are as follows.

"Real" reference	Correction temperature	Temperature factor
0°C	0.1°C	+0.1
37°C	36.9°C	+0.1
56°C	55.9°C	+0.1

Procedure 24

Wright-Giemsa stain, rapid modified

Principle

The Wright-Giemsa stain is available in a modification that requires only 1 to 3 minutes. This neutral dye is a combination of basic thiazine dyes and acid eosin that attach to oppositely charged sites on proteins. The results are metachromatic.

Materials

Wright-Giemsa stain
Absolute methanol
Phosphate buffer, pH 6.0

Application

Wright-Giemsa (modified) is a rapid stain for smears and imprints to stain background materials and cells and a wide variety of microorganisms.

Precautions

Avoid getting reagents in eyes or on skin or clothing; if this occurs, flush with copious quantities of water. Use with adequate ventilation. If stain is discarded into the sink, flush with large volumes of water to prevent azide buildup, which can react with lead and copper plumbing to form highly explosive metal azides.

Procedure

1. Prepare smear or imprint.
2. Fix slide by dipping it into absolute methanol for 15 seconds. After 15 seconds, allow excess to drain.
3. Dip slide in Wright-Giemsa stain for 1 minute. Allow excess to drain.
4. Rinse slide in phosphate buffer for 5 minutes without agitation.
5. Rinse slide briefly with running distilled water.
6. Allow to air-dry. Examine.

Note: The staining intensity may be altered by increasing or decreasing dips in the Wright-Giemsa stain. Reagents and procedures vary slightly by manufacturer. Be sure to follow the protocol on the package insert.

Results

Blood cells stain pink as with the Wright stain. The cytoplasm is basophilic. The chromatin of white blood cells is purple. Bacteria are blue. Parasitic protozoan nuclei are red.

PROCEDURE 25

X and V factor requirement

Purpose

To differentiate *Haemophilus* spp. based on their requirement for X and/or V factors

Principle

A suspension of bacteria to be tested is lawned onto the surface of a minimal medium. Disks or strips impregnated with X, V, and X and V factors are placed on the surface of the medium. After incubation, the pattern of bacterial growth determines which factor(s) the bacteria require.

Specimen

Isolated colonies on chocolate agar

Medium

Trypticase soy broth
Mueller-Hinton or trypticase soy agar plate
X, V, and X and V factors

Procedure

1. Make a suspension of the isolate in trypticase soy broth. The suspension is incubated at 35° C for about 2 hours to exhaust any X and V factors carried over from the plating medium.
2. A swab is used to inoculate the broth onto plated medium devoid of X and V factors; Mueller-Hinton or trypticase soy agars are acceptable.

3. After the plates dry, sterile forceps are used to place a strip or disk containing X factor gently on the plate. A V factor strip or disk is placed parallel to the X factor strip or disk approximately 15 mm away. A greater distance away, a strip or disk containing both X and V factors is placed onto the medium.
4. The plate is incubated at 35° C in 5% to 10% CO₂ for 18 to 24 hours. The plates are then examined for growth around and/or between the strips.

Interpretation

V factor only required: Growth entirely around the V factor disk or strip and the disk or strip containing both X and V factors

X factor only required: Growth entirely around the X factor disk or strip and the disk or strip containing both X and V factors

X and V factors both required: Growth between the X and V disks or strips and growth around the disk or strip containing both X and V factors

Controls

V factor only required: *Haemophilus parainfluenzae*

Both X and V factors required: *Haemophilus influenzae*

Glossary

5' nuclease assay Real-time polymerase chain reaction (PCR) detection technique; also called the *Taqman assay*. This assay uses a probe labeled on the 5' end with a fluorophore and on the 3' end with a quencher. The probe binds to target DNA and is dissociated by the action of DNA polymerase when primer extension occurs. The dissociation of the probe results in fluorescence because the fluorophore and quencher are removed from close proximity.

A

accuracy Degree of conformity of a measurement to a standard or a true value.

achlorhydria Absence of hydrochloric acid from the gastric juice.

acid-fast See **acid-fastness**.

acid-fast bacteria Microorganisms that are resistant to the removal of carbolfuchsin stain with acidified alcohol are designated as acid-fast bacteria. Mycobacteria and related species with waxes and phospholipids in their cell walls share this feature.

acid fastness Ability of bacteria, such as the *Mycobacterium* spp., to retain dye when treated with mineral acid or an acid-alcohol solution.

acquired immune system See **adaptive immunity**.

acquired immunodeficiency syndrome (AIDS) The most severe clinical manifestation of the disease spectrum caused by human immunodeficiency virus (HIV) infection.

acquired resistance See **adaptive immunity**.

actinomycosis (pl. actinomycoses) Chronic, granulomatous, infectious disease characterized by the development of sinus tracts and fistulae that erupt to the surface and drain pus containing sulfur granules. It is caused by *Actinomyces israelii* and related anaerobic organisms.

actinomycotic mycetoma Mycetoma caused by bacteria belonging to the group actinomycetes.

acute glomerulonephritis Poststreptococcal disease that occurs after a cutaneous or pharyngeal infection that results from antigen-antibody complexes deposited in the glomeruli.

acute otitis media. See **otitis media**.

acute phase Early stage of a disease preceding the adaptive phase of the immune response.

acute urethral syndrome Condition in which patients experience dysuria and frequency with bacterial urine colony counts fewer than 10^5 organisms/mL of urine.

adaptive immune response See **adaptive immunity**.

adaptive immunity Immune response elicited by a specific stimulus from a foreign molecule that causes immunogen recognition by B, T-helper, or cytotoxic T cells and results in the proliferation and differentiation of the stimulated cells into effector cells and memory cells; an immunity resulting

from a previous encounter of the host and an immunogenic stimulus.

adenylate cyclase toxin Virulence factor of *Bordetella* spp. that inhibits host epithelial and immune effector cells by inducing supraphysiologic concentrations of cyclic adenosine monophosphate.

adhesin Thin, filamentous, protein structures, including proteinaceous capsular antigens (fimbrial antigens), that mediate the adhesion of bacteria to surfaces and play a role in pathogenesis. They have a high affinity for various epithelial cells.

aerobe Microorganism that lives and grows in the presence of oxygen or requires oxygen.

aerobic respiration Process of cellular respiration utilizing oxygen to produce energy.

aerotolerance test Test used to determine whether an isolate is a strict anaerobe or a facultative anaerobe.

aerotolerant anaerobe Microorganism that grows best in the absence of oxygen but can tolerate low concentrations of oxygen.

agar dilution minimal inhibitory concentration (MIC) Assay in which a specific concentration of antimicrobial agent is incorporated into an agar medium contained in a Petri plate to determine the lowest concentration of the assayed antimicrobial agent that inhibits visible growth of the bacterium being tested. As many as 36 bacterial isolates from individual patients and quality control strains are tested on a series of agar dilution plates, each containing a different concentration of antimicrobial agent.

agarose gel electrophoresis Method used to separate nucleic acid molecules. Agarose is made from seaweed and acts as a molecular sieve. Nucleic acids migrate through the agarose gel when an electrical current is applied. Nucleic acids are naturally negatively charged and will migrate to a positive pole separating the molecules based on mass and charge.

amastigote Life-cycle stage found in humans that is characteristic of blood and tissue flagellates. It is a small, oval, intracellular body with a prominent nucleus and small kinetoplast.

aminoglycoside An antibiotic that has a six-membered ring with amino group substituents, aminocyclitol. The term *aminoglycoside* is so named because of the glycosidic bonds between the aminocyclitol and two or more amino-containing or non-amino-containing sugars.

δ -aminolevulinic acid (ALA) Substrate used in biochemical testing of *Haemophilus* spp. to determine X factor (hemin) requirement.

amorphous debris Debris without form or shape that is made up of necrotic tissue or cells, protein fluids, or mucus.

amplicon Amplified DNA that results from several cycles of the polymerase chain reaction (PCR); also known as a *PCR product*.

- anaerobe** Organism that does not require oxygen for life and reproduction.
- anaerobic chamber** Incubation system that provides an oxygen-free environment for inoculating media and incubating cultures.
- anaerobic transport system** Systems designed to maintain the viability of anaerobic bacteria from the time of collection to processing of the specimen. The vials are gassed out with oxygen-free carbon dioxide or nitrogen and contain an oxygen tension indicator.
- analytic activity** Process of analyzing a sample.
- analytic sensitivity** Ability of a test to detect a particular analyte or a small change in its concentration.
- analytic specificity** Ability of a test to detect substances other than the analyte of interest.
- anamnestic immune response** Stronger, quicker humoral or cell-mediated response on subsequent exposure to an immunogen.
- anamnestic response** See **anamnestic immune response**.
- anamorph** Form of a fungus that reproduces asexually.
- anneal** Pairing, using hydrogen bonding, of complementary RNA and DNA sequences to form a double-stranded molecule; describes the binding of a short probe or primer.
- annealing temperature (Ta)** The optimal temperature at which two complementary strands of nucleic acid form a double-stranded molecule.
- antagonism** Occurs when the antimicrobial activity of a combination of antimicrobial agents is less than the activity of the individual agents alone.
- anthrax** Disease of livestock with which humans are accidentally infected. In humans, it can take four clinical forms: cutaneous, gastrointestinal, injectional, and inhalational (pulmonary anthrax); an infection associated with *Bacillus anthracis*.
- antibiogram** Compilation of selected microorganisms and the percentage susceptibility to selected antimicrobial agents.
- antibiotic** Substance produced by a microorganism used to prevent or treat infection caused by bacteria and other pathogenic microorganisms.
- antibiotic stewardship** The effort to measure and improve how antimicrobial agents are prescribed by primary care providers and used by patients.
- antibody** Protein, which in the monomer form has two heavy and two light chains, consisting of a hypervariable region capable of recognizing a particular binding site on another molecule; an immunoglobulin molecule characterized by a specific amino acid sequence produced by the host as a result of a specific immunogenic stimulation.
- antibody titer** Reciprocal of the highest dilution of a clinical sample that can be detected in a reaction with the corresponding antigen.
- anticodon** Triplet of bases on the tRNA that binds the triplet of bases on mRNA. It identifies which amino acid will be in a specific location in the protein.
- antigen** A molecule that exhibits reactivity with an immunologic effector, such as an antibody or T-cell receptor.
- antigenic drift** Phenomenon of slight antigenic change seen in influenza viruses over time as a result of minor mutations in the ssRNA genome.
- antigenic shift** Phenomenon whereby an often unexpected change occurs in influenza virus strains. This antigenic change is often so drastic that it triggers pandemics.
- antigenic variation** Systematic change in surface antigens while organisms such as *Borrelia recurrentis* are in the host during the course of a single infection.
- antimicrobial agent** Substance used to prevent or treat infection caused by bacteria and other pathogenic microorganisms.
- antimicrobial assay** Method to measure the amount of antimicrobial agent in serum or body fluid.
- antimicrobial pressure** Antimicrobial use induces selection pressure, allowing only the fittest and least susceptible bacterial populations to thrive and thereby displace susceptible populations; also referred to as *antimicrobial selection pressure*.
- antimicrobial removal device (ARD)** Resin that nonspecifically absorbs any antimicrobial agent present in the patient's blood.
- antiseptics** Destruction of microorganisms to prevent infection.
- antiseptic** Substance that inhibits, destroys, or reduces the bacterial load of living tissues. No sporicidal activity is implied; used specifically for substances applied to living tissue.
- antiseptic drug** Considered to be a representative germicide, except in the case of a drug purporting to be, or represented as, an antiseptic for inhibitory use as a wet dressing, ointment, dusting powder, or other use that involves prolonged contact with the body.
- appliqué form** Parasite at the edge of a red blood cell.
- arbovirus** Virus transmitted between vertebrate hosts by arthropods.
- archaea** Group of living microorganisms that exist in extreme conditions, such as in the absence of oxygen; in bodies of highly concentrated salt water; in hot, acidic waters of sulfur springs; and at temperatures near 80° C and pH levels as low as 2. They are classified as prokaryotes but are distinct from bacteria.
- arthroconidium** Asexual spore formed by the breaking of a hypha at the point of septation. Arthroconidia can be adjacent or separated by a disjunct cell.
- asaccharolytic** Microorganism that is unable to metabolize carbohydrates in the presence or absence of oxygen and that must rely on other carbon sources for energy.
- ascospore** Sexual spore produced by fusion of a male nucleus into a female cell in an ascus.
- ascus** Saclike structure usually containing two to eight ascospores.
- aseptic meningitis** Syndrome characterized by signs and symptoms of meningeal inflammation with the presence of inflammatory cells in the cerebrospinal fluid without bacteria or fungi recovered from cultures; often associated with meningitis caused by viruses.
- asexual reproduction** Reproduction by methods such as fission, in which the nucleus and cytoplasm are split to form two new, separate organisms.
- Asiatic cholera** See **cholera**.
- aspiration pneumonia** Pulmonary inflammation resulting from the abnormal entry of fluid, particulate substances, or endogenous secretions into the lower airways.

asymptomatic bacteriuria (ASB) The presence of bacteria in the urine without any symptoms of a urinary tract infection.

atypical pneumonia See **primary atypical pneumonia**.

auramine/auramine-rhodamine fluorochrome

stains Fluorochrome stains (auramine or auramine-rhodamine) are used to visualize acid-fast bacteria using a fluorescence microscope. Acid-fast bacteria appear yellow or orange.

autotroph Organism that produces organic compounds from carbon dioxide as a carbon source, using light or inorganic chemical compounds as a source of energy.

avibactam A non- β -lactam β -lactamase inhibitor used in combination with ceftazidime.

avidity Strength of binding between an antibody molecule and an antigen with multiple epitopes.

axostyle An axial rod present in several intestinal flagellates providing support.

B

Babès-Ernst granules Metachromatic granules of *Corynebacterium diphtheriae* that stain more intensely than other parts of the bacterial cell.

bacillary dysentery Gastrointestinal disease caused by bacterial infection. Symptoms include severe diarrhea, fever, stomach pain, nausea, and vomiting.

bacteremia Presence of viable bacteria in the blood, as evidenced by their recovery in blood cultures.

bacteria Also referred to as *eubacteria*; single-celled procaryote, microorganisms that lack a true cell nucleus and multiply by binary fission.

bacterial interference Potential pathogens inhibited by the nonpathogenic resident microbiota.

bacterial vaginosis (BV) Syndrome characterized by vaginal symptoms that include perivaginal irritation, vaginal odor described as fishy, and mild to moderate discharge. The condition is regarded as being polymicrobial.

bactericidal Antimicrobial that kills a microorganism.

bactericidal effect Killing effect or amount of antimicrobial agent required to kill a population of bacteria.

bacteriocin Proteins produced by some bacteria that inhibit the growth of other strains of the same organism or related species. Genes for bacteriocins may reside on plasmids.

bacteriophage Virus that infects bacteria.

bacteriostatic Antimicrobial that inhibits bacterial growth while present but does not kill the bacteria.

bacteriuria Presence of bacteria in the urine.

balanitis Inflammation of the glans (head) of the penis.

bamboo rods Term that describes the microscopic appearance of *Bacillus anthracis* in a Gram-stained smear, referring to the cells' arrangement and the unstained central spore.

baseline data Surveillance data that represent the normal endemic level of infections in a health care setting; used to gauge when an outbreak occurs.

Bayes theorem Formulas for calculating positive and negative predictive values are commonly referred to as *Bayes theorem*, published posthumously in 1763.

benchmarking Reference point obtained by using an industry's or profession's best practices to imitate and improve processes.

bias Mean difference of test results from an accepted reference method by systemic errors.

bile solubility Test that determines the ability of bacterial cells to lyse in the presence of bile salts; it is used to differentiate *Streptococcus pneumoniae* (bile-soluble) from other α -hemolytic streptococci (bile-insoluble).

biocrime Refers to a situation in which a biological agent is used to kill or make ill a single individual or small group of individuals.

biofilm Community of microorganisms attached to a solid surface.

biofouling Undesirable accumulation of microorganisms, plants, algae, and/or animals on wetted structures. For example, biofilms can form in pipes carrying drinking water, making the water unsafe to drink.

biological agent Any microorganism (including, but not limited to, bacteria, fungi, rickettsiae, or protozoa), or infectious substance (e.g., virus), or any naturally occurring, bioengineered, or synthesized component of any such microorganism or infectious substance, capable of causing the following: death, disease, or other biological malfunction in a human, animal, plant, or other living organism; deterioration of food, water, equipment, supplies, or material of any type; or deleterious alteration of the environment.

biological risk assessment Assessment of biological hazards.

biological safety cabinet (BSC) A station for the safe handling of aerosols and particulates; provides laboratory personnel protection.

biological warfare Deliberate use of bacteria, viruses, fungi, or toxins to injure people, animals, or crops to gain a military advantage or for political gain.

biorisk The risk associated with biological materials and/or infectious agents.

biorisk management The effective management of risks posed by working with infectious agents and toxins in laboratories.

biosafety The use of specific practices, safety equipment, and specially designed buildings to ensure that workers, the community, and the environment are protected from accidental exposure or unintentional release of infectious agents, toxins, and other biological hazards.

biosafety cabinet See **biological safety cabinet**.

biosafety level Maintenance of safe conditions in the laboratory environment to prevent harm to workers, nonlaboratory organisms, or the environment. The Centers for Disease Control and Prevention has established guidelines for four biosafety levels.

biosecurity Procedures intended to protect humans or animals against disease or harmful biological agents.

biosurveillance Systematic observation and data gathering to detect and counter biological threats to human or animal health, in order to achieve early warning and identification of such health threats, and general situational awareness of disease activity.

bioterrorism Intentional use of biological agents (bacteria, viruses, or toxins) to cause illness or death as an act of terrorism.

biothreat The threat posed by a harmful biological agent.

biotin-streptavidin system Immunologic assay based on the interaction of biotin with streptavidin. Antibody is linked to biotin. If an antigen specific to the antibody is present in a clinical specimen, it will bind to the antibody. After a wash step, streptavidin linked to a label (e.g., enzyme or fluorochrome) is added. Biotin and streptavidin will bind and the signal is measured.

bipolar staining Refers to the distinctive safety pin appearance that results from staining with a polychromatic stain such as Wayson or Wright-Giemsa stain.

black death Another name for plague, a disease caused by *Yersinia pestis*.

blackwater fever Syndrome associated with *Plasmodium falciparum* infection characterized by reddish to black urine resulting from hemoglobinuria.

blastoconidium Conidium formed by budding yeast along the hypha or pseudohypha or from a yeast cell.

blepharitis Inflammation of the eyelids.

bloodborne pathogen Pathogenic microorganism that is present in human blood and can cause disease in humans. These pathogens include, but are not limited to, hepatitis B virus and human immunodeficiency virus.

borderline oxacillin-resistant isolates Subtle type of oxacillin resistance in *Staphylococcus aureus* that lack the *mecA* gene and have oxacillin minimal inhibitory concentrations (MICs) right above (or zones of inhibition right below) the breakpoint for susceptibility.

borderline oxacillin-resistant *Staphylococcus aureus* (BORSA) See **borderline oxacillin-resistant isolates**.

Bordet-Gengou potato infusion agar Medium supplemented with glycerol, peptones, and horse or sheep blood for isolation of *Bordetella* spp.

botulinum toxin Neurotoxin primarily produced by *Clostridium botulinum*.

botulism Serious form of food poisoning caused by the ingestion of preformed botulinum toxin produced by *Clostridium botulinum*.

bradyzoite *Toxoplasma gondii* life-cycle stage that forms within a cystlike structure. The organisms multiply slowly but may be transformed into tachyzoites when the host's immune system is impaired.

brain abscess Swollen area in the brain tissue containing pus caused by inflammation and collection of infected material coming from a local infectious source (e.g., ear infection, infection of the paranasal sinuses, infection of the mastoid air cells of the temporal bone, epidural abscess) or remote infectious source (e.g., lung, heart, kidney).

branched DNA (bDNA) assay Technique that amplifies a signal after probes have annealed to target nucleic acid; a capture probe attached to a solid support anneals to target nucleic acid. Target probes also anneal to the nucleic acid and to preamplifier probes. Amplifier probes then bind to the preamplifier probes, resulting in the formation of branched DNA (bDNA) structures. Label probes then bind to the bDNA structures, resulting in massive signal amplification.

breakpoint Minimal inhibitory concentration or zone diameter value used to indicate susceptible, intermediate, or resistant for an antimicrobial agent, as defined by the interpretive criteria used in Clinical and Laboratory Standards Institute standards.

breakpoint panel Variation of the standard broth microdilution minimal inhibitory concentration panel is the breakpoint panel, in which only one or a few concentrations (around the cut off between sensitive and resistant) of each antimicrobial agent are tested on a single panel.

Brill-Zinsser disease Reactivation of *Rickettsia prowazekii* in patients who have had epidemic typhus. The reactivation disease is normally milder than epidemic typhus; also called *recrudescence typhus*.

brittle Adjective used in defining colony consistency; splinters.

bronchiolitis An infectious disease usually of infants and young children usually caused by respiratory viruses, characterized by inflammation of the bronchioles and clinically expressed as a febrile upper respiratory tract infection, with concurrent signs of lower respiratory tract airway obstruction.

bronchitis Illness characterized by inflammation of the bronchi; clinically expressed as cough, usually with sputum production, and evidence of concurrent upper airway infection.

bronchoalveolar lavage Procedure in which saline or some other physiologic fluid is instilled into the alveoli and recovered for the purpose of culture or cytology examination.

broth macrodilution minimal inhibitory concentration (MIC) Broth dilution MIC tests performed in test tubes are referred to as *broth macrodilution MIC* or *tube dilution MIC* tests. Generally, a twofold serial dilution series, each containing 1 to 2 mL of antimicrobial agent, is prepared.

broth media Liquid medium used as a supplement to agar plates to detect small numbers of most aerobes, anaerobes, and microaerophiles.

bubo (pl. buboes) Inflammation of a lymph node, characterized by swelling and ulceration, resulting from infection, especially in the area of the armpit or groin, that is characteristic of certain infections such as bubonic plague; inflamed, tender swelling of a lymph node.

bubonic plague Most common form of plague, which usually results from the bite of a flea infested with *Yersinia pestis*. A common result is the formation of a bubo.

buffered charcoal yeast extract (BCYE) Recommended medium for isolation of *Legionella* spp. It should be supplemented with α -ketoglutarate.

bullae (pl. bullae) Blister larger than 5 mm (about ¼ inch) in diameter with thin walls that is full of fluid.

bullous impetigo Form of impetigo in which the skin lesions are bullae instead of vesicles. The crusts are thin and greenish yellow. Exfoliative toxin has been implicated in bullous impetigo. See **impetigo**.

Buruli ulcer Infectious disease caused by *Mycobacterium ulcerans* characterized by painless swelling that later develops into a lesion.

butyrous Adjective used in defining colony consistency; creamy.

C

CAMP test CAMP stands for Christie, Atkins, and Munch-Peterson, a test used for the presumptive identification of group B streptococci (*Streptococcus agalactiae*). A positive result is determined by enhanced, arrowhead, β -hemolysis from the interaction of CAMP factor from group B

- Streptococcus* and the β -lysine from certain strains of *Staphylococcus aureus*.
- canaliculitis** Inflammation of the canaliculus, thin channel in bone.
- capnophile** Microorganism that grows best in the presence of carbon dioxide.
- capnophilic** Term used to describe microorganisms that require an increased concentration of CO₂, usually between 5% and 10%.
- capsid** Protein covering a virus.
- capsule** Structure in some prokaryotic cells located outside the cell wall of bacteria. It is usually made up of polysaccharides but could be composed of other materials; used by microorganisms as a protective structure against phagocytosis and physical and chemical effects of the environment.
- carbapenemase-producing Enterobacterales (CPE)** Member of the order Enterobacterales that produces a carbapenemase, a β -lactamase able to hydrolyze several antibiotics, including penicillins, cephalosporins, monobactams, and carbapenems.
- carbuncle** Cluster of furuncles caused by the subcutaneous spread of a staphylococcal infection.
- carrier** Individual or animal that harbors a potentially pathogenic organism or infectious agent without the host showing signs of the disease, but that serves as a source of infection to susceptible individuals.
- carrier state** Condition in which an individual or animal harbors a potentially pathogenic organism or infectious agent without the host showing signs of the disease, but that serves as a source of infection to susceptible individuals.
- Cary-Blair transport media** Semi-solid medium used for the maintenance of various pathogens, including enteric bacteria such as *Salmonella*, *Shigella*, and *Campylobacter* spp. in stool specimens.
- case definition** Description establishing parameters that define a case in an outbreak investigation.
- catalase** Breaks down hydrogen peroxide to water and oxygen. Useful for distinguishing staphylococci (positive) from streptococci (negative).
- catarrhal phase** Initial phase of pertussis. Symptoms are nonspecific and include sneezing, mild cough, runny nose, and sometimes conjunctivitis.
- category A agents** Bacteria, viruses, and toxins that, if used as bioterror agents, would cause significant public health disruption.
- catheter-associated urinary tract infection (CAUTI)** Urinary tract infection linked to the use of a urinary catheter.
- catheter-related bloodstream infection (CRBSI)** Bloodstream infection related to the presence of an intravascular device in a major vessel, generally ending at or near the heart.
- cell culture** Cells removed from an organism and grown in a complex medium for several purposes, including growth of viruses.
- cell wall-deficient** Refers to gram-positive or gram-negative bacteria lacking a cell wall; also called *L forms*. Exposure to antimicrobial agents that inhibit cell wall synthesis (e.g., penicillin) can lead to this condition. The mycoplasma inherently lack the ability to produce a cell wall; therefore when they were first discovered, they were referred to as *cell wall-deficient*.
- cell-mediated immune response** Adaptive immune response that involves activation of macrophages and natural killer cells; production and release of antigen-specific, cytotoxic T lymphocytes; and release of various cytokines in response to an immunogenic stimulus. It does not involve antibodies.
- cellulitis** Serious, sometimes life-threatening, skin infection that involves deep tissues; can be accompanied by bacteremia or sepsis.
- Centers for Disease Control and Prevention (CDC)** A federal agency established for the protection of the health and safety of people, at home and abroad, that provides information to enhance health decisions and health promotion.
- central line-associated bloodstream infection (CLABSI)** One of multiple acute-care, hospital-acquired infections; occurs as a result of instrumentation, increased use of antimicrobial agents, breaks in aseptic technique, and lack of hand hygiene.
- central nervous system (CNS)** The largest part of the nervous system; made up of the brain and the spinal cord.
- cercaria** Tailed stage in the life cycle of flukes that is produced from miracidia that develop in snail tissue. This stage is released into the water.
- cerebrospinal fluid (CSF)** Clear fluid formed by the choroid plexus or ventricles that fills the subarachnoid space in the brain and serves as a cushion for the cortex.
- cervicitis** Inflammation of the tissues surrounding the cervix; can be caused by infection by a variety of agents, such as *Chlamydia trachomatis* and *Neisseria gonorrhoeae*.
- cervicofacial actinomycosis** Infectious disease caused by the anaerobic *Actinomyces*, which can occur in the mouth, lungs, and digestive tract. Abscesses may penetrate the surrounding bone and muscle to the skin, where they break open and leak large amounts of pus. When it occurs in the face or neck, it is termed *cervicofacial*.
- chancre** See *syphilitic chancre*.
- chancroid** Sexually transmitted disease (infection) caused by *Haemophilus ducreyi*.
- chemiluminescent immunoassay (CLIA)** Type of labeled assay when a chemical reaction emits light.
- chemotactic agent** Protein that is able to direct the migration of a specific cell.
- chemotaxis** Movement of cells in response to a chemical stimulant.
- chlamydia** The most commonly reported infectious disease in the United States. Chlamydia is caused by the obligate intracellular bacterium *Chlamydia trachomatis*, which has a biphasic developmental cycle.
- chlamydiosis** Infection caused by *Chlamydia* spp.
- cholera** Acute, infectious, diarrheal illness caused by *Vibrio cholerae*, which may occur in epidemic proportions.
- cholera toxin** Powerful enterotoxin produced by *Vibrio cholerae*; also referred to as *cholera toxin*.
- cholera toxin** See *cholera toxin*.
- chromatoidal bar** RNA that has been condensed into a barlike structure within the cyst of some protozoan organisms.
- chromoblastomycosis** See *chromomycosis*.
- chromogenic substrate** Molecule that is initially colorless; when cleaved by a specific enzyme, it becomes a colored compound.

chromomycosis Chronic fungal infection of the skin and subcutaneous tissues, also called *chromoblastomycosis*. The most commonly associated fungi are *Fonsecaea pedrosoi*, *Phialophora verrucosa*, *Cladophialophora carrionii*, and *Fonsecaea compacta*.

chromosomally mediated resistant *Neisseria gonorrhoeae* (CMRNG) Type of penicillin resistance in *N. gonorrhoeae* attributable to genes located on the chromosome that code for an alteration in penicillin-binding proteins.

-cidal Suffix used to indicate that a substance destroys microorganisms (e.g., bactericidal, virucidal).

ciguatera Form of food poisoning caused by ciguatera toxin produced by dinoflagellates; passed up the food chain; ingested in snapper, sea bass, or grouper.

citrate test Bacteriologic test to determine an organism's ability to use citrate as a sole carbon source.

Clark and Lub's medium Classic bacteriologic medium used for the methyl red, Voges-Proskauer test.

clavulanic acid A β -lactamase inhibitor often used with amoxicillin.

clean-catch midstream urine specimen A urine sample collected using a technique that reduces contaminant skin biota; the patient cleanses the external genitalia and begins voiding urine, collecting the sample midstream.

Clinical and Laboratory Standards Institute (CLSI) International, interdisciplinary, nonprofit, standards-developing, educational organization that promotes the development and use of voluntary consensus standards and guidelines in the health care community.

Clinical Laboratory Improvement Amendments (CLIA) Law first released in 1988, with subsequent amendments, establishing quality standards for all laboratory testing to ensure accuracy, reliability, and timeliness of patients' test results.

clinical (diagnostic) sensitivity Proportion of positive test results obtained when a test is applied to patients known to have a disease.

clinical (diagnostic) specificity The proportion of negative results obtained when a test is applied to patients known to be free from a disease.

***Clostridioides difficile*-associated disease** Disease characterized by damage to the lining of the colon; caused by *Clostridioides difficile*; also referred to as pseudomembranous colitis.

clumping factor Cell-bound coagulase that is able to agglutinate cells in plasma; may be used to screen for *Staphylococcus aureus*.

coagglutination Particulate agglutination assay using *Staphylococcus aureus* to bind the Fc portion of antibodies.

coagulase Clotting enzyme (staphylocoagulase) useful in differentiating coagulase-positive staphylococci, such as *Staphylococcus aureus*, from coagulase-negative staphylococci.

coagulase-negative staphylococci (CoNS) Staphylococci that lack the enzyme coagulase.

codon Gene sequence inscribed in DNA and RNA composed of trinucleotide units.

cold-agglutinating antibody Antibody that agglutinates antigens at 4° C; if present, suggestive of infection caused by *Mycoplasma pneumoniae*.

cold enrichment Method of incubating broth cultures at 4° C for several days to select for bacteria, such as *Listeria* and *Yersinia* spp., that grow well at this temperature.

College of American Pathologists (CAP) The principal organization of board-certified pathologists that serves and represents the interest of patients, pathologists, and the public by fostering excellence in the practice of pathology and laboratory medicine.

colonization Formation of a population of microorganisms in the host that does not cause disease.

colony-forming unit (CFU) Number of microbes that grow from a measured inoculation; expressed as CFU/mL. Bacteria are easily seen in direct smears at concentrations of 10×5 CFU/mL following cyto centrifugation.

colony morphology Colony characteristics and form of microorganisms grown on solid media.

commensal Refers to an organism that lives in a relationship in which one organism (commensal) derives food or other benefits from another organism (host) without hurting or helping the host.

commensalism Relationship between different species in which one (commensal) benefits from the other (host) without causing harm. The host species does not benefit from the relationship or organism.

communal living Community living program; might include prisons and behavioral health facilities. In these facilities, infections might be found that are spread by contact (illicit tattooing) or by intimate contact with blood and body fluids.

community-acquired bacteremia Bacteremia that occurs in individuals living in the general community.

community-acquired infection Infection that starts in the community and is not acquired in a health care setting.

community-acquired pneumonia (CAP) A pneumonia acquired from a community source as opposed to in a health care facility.

community-associated, methicillin-resistant *Staphylococcus aureus* (CA-MRSA) Infections caused by MRSA that are acquired in the community. CA-MRSA infections and outbreaks have been reported among athletes, correctional facility inmates, military recruits in close-contact environments, pediatric patients, and tattoo recipients. See also **methicillin-resistant *Staphylococcus aureus* (MRSA)**.

competency Ability to apply relevant knowledge, skills, and abilities to successfully perform all assigned duties.

competent As in an immunocompetent patient, refers to a state of well-being in which the immune system is functioning properly and is able to protect the individual from infectious diseases. As in genetics, a cell, such as a bacterium, able to take in DNA fragments from the environment.

competitive immunoassay A labeled antibody or antigen competes with an unlabeled antibody or antigen from a patient. Signal is inversely proportional to the analyte concentration.

complement system Series of plasma proteins that functions as an enzyme or as binding protein; plays an essential role in host defense against infectious agents and in the inflammatory process.

complement deficiency Lack of any one or more of the proteins that make up the biochemical cascade of the complement system.

complement fixation (CF) Activation of complement proteins. Also an immunoassay method using complement proteins as a means to detect immunocomplexes.

condyloma acuminatum (pl. acuminata) Anogenital warts caused by human papillomavirus.

condylomata lata Flat, mucoid, fleshy, wartlike growths that often occur in the perianal region but can occur in other moist regions of the body; papules of secondary syphilis.

congenital infection Refers to being acquired in utero when the organism infecting a woman crosses the placenta to infect the fetus.

conidium Asexual fungal reproductive structure usually formed at the side of hyphae. It is not contained in a saclike structure; small and usually single-celled conidia are called *microconidia*, and larger multicelled conidia are called *macroconidia*.

conjugate Substance formed by combining two different molecules, specifically in immunologic assays, adding an enzyme or fluorochrome to an antibody or antigen.

conjugation Transfer of genetic material between bacteria through cell-to-cell contact.

conjunctivitis Inflammation of the conjunctiva (the mucous membrane that covers the front of the eye and lines the inside of the eyelids).

consistency Degree of a feature such as density, firmness, viscosity, and texture.

continuing education Educational learning activities that adults pursue postsecondary or after any formal education; may include seminars and one-time online courses and other forms of training.

continuous bacteremia Bacteremia (see earlier) that occurs when organisms are coming from an intravascular source and are consistently present in the bloodstream.

continuous cell culture Culture capable of a virtually unlimited number of passages, sometimes referred to as an *immortal cell culture*.

continuous quality improvement (CQI) Progressive incremental improvement of processes, safety, and patient care.

onalescent phase In pertussis, the third and final phase, generally beginning within 4 weeks of onset of infection. There is a decrease in the frequency and severity of the coughing spells; complete recovery occurs in weeks to months.

creamy See *butyrous*.

creeping eruption Rash caused by the movement of hookworm larvae beneath the surface of the skin.

cross-functional teams Teams composed of members from different departments.

cumulative antibiogram Report generated by analysis of individual susceptibility results on isolates from a particular institution in a defined period; represents the percentage of isolates of a given species susceptible to the antimicrobial agents commonly tested against the species.

Curschmann spirals Condensed bronchial secretions that take on the shape and size of bronchial passageways, particularly if there is constriction or obstruction.

customer concepts View or perception each laboratory customer holds in regard to quality and his or her own expectations.

cycling probe technology Technique that amplifies a signal after a DNA-RNA-DNA chimeric probe is incubated with a target nucleic acid. The probe has a 5' fluorophore and a 3' quenching molecule; the result is no fluorescence until the probe anneals to the target nucleic acid. RNase H digests the RNA in the probe after annealing has occurred, resulting in fluorophore release from the quencher. This results in an increase in fluorescent signal. A new probe molecule will then anneal to the same target nucleic acid, and the process repeats for more fluorescence.

cyst Infective form of a protozoan; characterized by the formation of a thick protective wall that is resistant to environmental factors.

cysticercus Larval stage of *Taenia* spp.; fluid-filled sac that contains the scolex of the tapeworm.

cystine-tellurite blood agar (CTBA) Selective and differential medium for the recovery of *Corynebacterium diphtheriae* and related organisms.

cystitis Inflammation of the urinary bladder.

cytocentrifugation Mechanical process in which centrifugal force is used in an instrument to deposit cells on a glass slide for staining and viewing.

cytolytic toxins Exotoxins that can affect erythrocytes, leukocytes, macrophages, and platelets.

cytopathic effect (CPE) Visible changes in cells resulting from toxins or virus infection.

cytostome A mouthlike opening present in certain protozoa.

D

D-zone test Test used to detect inducible clindamycin resistance in staphylococci and β streptococci. An erythromycin disk is placed next to a clindamycin disk on a standard disk diffusion plate; flattening of the clindamycin growth inhibition zone between the two disks indicates inducible clindamycin resistance.

dacryoadenitis Infection of the lacrimal gland.

dacryocystitis Infection of the lacrimal sac.

darting motility Distinct type of rapid motility, characteristic of *Campylobacter* spp., observed under a wet preparation; results from their long polar flagellum.

data mining Computer-based search of data to indicate areas of potential concern for an infection control practitioner; implies in-depth correlation of computer data.

deaminase Enzyme that removes amine groups (NH_2) from amino acids.

decarboxylase Enzyme capable of removing a carboxy group (CO_2) from an amino acid.

decolonization Process to reduce the population of certain bacteria on select patient populations, such as patients in intensive care units.

definitive host Individual in which a parasite has its adult and/or sexual reproductive stage.

denaturation Dissociation of double-stranded nucleic acid molecules into single strands. Denaturation is usually accomplished in molecular biology techniques with high heat (-95°C) so primers or probes may then anneal to target nucleic acid sequences.

dendrogram Branching, treelike diagram frequently used to show the interrelationships among a group of organisms.

density Degree of opacity of a substance or medium that transmits light.

deoxynucleotide triphosphate (dNTP) Individual nucleic acid base used by enzymes such as DNA polymerase to synthesize new nucleic acid strands. Adenine (dATP), cytosine (dCTP), guanine (dGTP), and thymidine (dTTP) are used by DNA polymerase to synthesize new DNA strands.

dermatophyte Fungus able to use keratin and infect the skin, hair, and nails of humans; belongs to the genera *Epidermophyton*, *Microsporum*, and *Trichophyton*.

dermatophytosis Infection caused by a dermatophyte.

detection limit Analytic sensitivity is usually defined at the 0.95 confidence level (± 2 standard deviations); may be referred to as the detection limit. In microbiology, the detection limit may be correlated to the number of colonies in the culture or to the lowest quantity of antigen or antibody a test can detect.

diapedesis Passage of inflammatory cells and other formed elements in the blood through the endothelial walls of the blood vessel.

differential media Media that allow grouping of microbes based on different characteristics demonstrated on the medium (i.e., colony morphology).

differential stain The Gram stain colorizes cells differently so each component can be recognized (gram-positive cell wall, gram-negative cell wall).

diffusely adherent *Escherichia coli* (DAEC) Enteropathogenic and uropathogenic *E. coli* strains defined by their attachment to HEp-2 cells.

dimorphic fungi Fungi that exist as a yeast, spherule, or mold, depending on growth conditions.

diphtheria Disease caused by *Corynebacterium diphtheriae*.

diphtheria toxin Potent toxin produced by *Corynebacterium diphtheriae* that causes tissue necrosis, exudate formation, and systemic effects involving the heart, kidneys, and nervous system.

diploid Containing two copies of each chromosome. Diploid is the normal genetic makeup for eukaryotic cells.

diploid cell culture Cells used for the growth of viruses that contain the normal number of chromosomes.

direct agglutination test Agglutination assay in which the antigen naturally occurs on a cell.

direct fluorescent antibody (DFA) test Assay using a fluorochrome-labeled primary antibody to detect an antigen in a clinical specimen or culture isolate.

direct microscopic examination Examination of the specimen under a microscope, with or without staining.

direct sandwich immunoassay Assay in which an antigen is captured by an antibody affixed to a solid phase. A second labeled antibody is added that is able to bind to the antigen.

disinfectant Substance designed to be used on inanimate objects to kill or destroy disease-producing microorganisms, including spores in some cases.

disinfection Reduction in the amount of microbes that may cause disease from an environment.

disseminated intravascular coagulation (DIC) Pathologic activation of blood-clotting mechanisms that occurs in response to a variety of disease states, which leads to the formation of small blood clots inside the blood vessels throughout the body.

dissemination Spread to other sites.

DNA microarray Technique used to assay the entire expression of genes in cells; also can be used to identify organisms from samples, mutations, and new genes, and for other purposes. Microscopic spots of single-stranded DNA are placed on a solid support in an array, and unknown samples are fluorescently labeled and hybridized to the DNA on the array. A scanner is used to identify hybrids and indicate positive samples or high levels of gene expression.

DNA polymerase Enzyme that synthesizes new strands of DNA. DNA polymerase uses deoxynucleoside triphosphates and primers to synthesize the new strands. The most common DNA polymerase in use in molecular diagnostics methods is *Taq* DNA polymerase, a thermostable enzyme that can withstand the denaturation temperatures used in polymerase chain reaction and other methods.

DNA replication Copying of a double-stranded DNA strand in a cell before cell division.

DNA transcription Process through which a DNA sequence is enzymatically copied by an RNA polymerase to produce complementary RNA.

DNase Enzyme capable of hydrolyzing double-stranded DNA.

Donovan bodies Characteristic intracellular organisms found in Giemsa or Wright stain of macrophages that can confirm the diagnosis of donovanosis. Donovan bodies measure 0.5 to 0.7 mm $\times$ 1 to 1.5 mm in diameter and may or may not be capsulated. See also **granuloma inguinale**.

double immunodiffusion An immunologic assay performed in agarose. Antigen is placed in a well near a well containing an antibody. The reactants diffuse toward each other. At the zone of equivalence, a precipitate is formed. Based on the similarity between the antigen and antibody, different precipitation patterns are formed.

double indirect fluorescent antibody Assay in which an antibody in a patient's serum binds to a known antigen. A second, unlabeled, antihuman antibody is added, and multiple molecules can bind to the patient's antibody. A third fluorochrome-labeled antibody directed against the second antibody is then added.

dual-probe fluorescence resonance energy transfer

(FRET) Real-time polymerase chain reaction (PCR) detection method that uses two probes: one with a donor fluorophore on the 3' end and the other with an acceptor fluorophore on the 5' end. The two probes anneal head-to-tail to accumulated PCR products. Light from the real-time PCR instrument excites the donor fluorescent dye, and this energy is transferred to the acceptor dye by FRET. The acceptor dye then gives off this energy, and it is read by the instrument. As the PCR amplicon accumulates, fluorescence increases.

dysgonic fermenter (DF) Gram-negative fermentative bacillus that has difficulty growing on routine media. At one time, these were referred to by the CDC as *DF-1*, *DF-2*, and *DF-3*; all have now been placed in the genus *Capnocytophaga*.

dysuria Difficulty or painful urination.

E

ecthyma gangrenosum Cutaneous infection most commonly associated with *Pseudomonas aeruginosa* bacteremia.

ectoparasite Organisms that live on, rather than in, the human body; include fleas, lice, ticks, and mites.

- edema factor (EF)** One of three proteins that make up the anthrax toxin. Edema results from the combination of protective antigens with this factor.
- efflux pumps** Proteins located in the bacterial cell membrane that transport molecules out of the cell.
- EI Tor** Strain of *Vibrio cholerae* belonging to the serotype O1.
- electroendosmosis** Movement of buffer ions toward the cathode causing antibody molecules, which have a weak net negative charge, to be carried toward the cathode.
- Elek test** Immunodiffusion test originally described by Elek for detecting the toxin produced by *Corynebacterium diphtheriae*.
- elementary body (EB)** Metabolically inactive infectious form of *Chlamydia*.
- elevation** Description of colony morphology. The elevation should be determined by tilting the culture plate and looking at the side of the colony. Elevation may be raised, convex, flat, umbilicate (depressed center, concave, an “inny”), or umbonate (raised or bulging center, convex, an “outy”).
- Embden-Meyerhof pathway** Energy-yielding pathway converting one molecule of glucose to two molecules of pyruvate; also called the *Embden-Meyerhof-Parnas pathway*.
- emergency response plan** Plan established by each health care setting to deal with the potential of some emergency, such as a bioterror event.
- emerging infection** Infection whose incidence has increased in number of spread geographically in the past few years and could increase in the near future.
- emerging pathogen** Pathogen that is newly recognized, has spread geographically, or has become more virulent.
- emerging zoonosis** Infectious agent that is transmitted from animals to humans and is a newly discovered agent, modified agent, or previously known agent that moves to a new geographic location.
- empiric antimicrobial therapy** Institution of treatment based on practical experience in the absence of culture data.
- employee right-to-know** Occupational Safety and Health Administration laboratory chemical hygiene plan and hazard communication standard (29 CFR 1910.1200, Hazard Communication Standard); states that all clinical laboratory personnel have a need and a right to know the hazards and identities of the chemicals to which they are exposed when working. They also need to know what protective measures are available to prevent adverse effects from occurring.
- empyema** Collection of purulent fluid in the pleural space between the lung and chest wall.
- encapsulated** Strains of bacteria and yeast that produce a capsule.
- encephalitis** Inflammation of the brain.
- endemic relapsing fever** Tickborne relapsing fever caused by *Borrelia* spp.
- endemic spirophilis** Nonvenereal spirochetal disease of the Middle East or Africa caused by *Treponema pallidum* subsp. *endemicum*. Clinical disease closely resembles yaws and is also known as *bejel*.
- endogenous (microbiota)** Originating from within an organism.
- endogenous anaerobe** Anaerobe that exists inside the body of an animal (member of the indigenous microbiota).
- endophthalmitis** Inflammation of the intraocular fluids and tissues.
- endotoxin** Lipopolysaccharide (LPS) component of gram-negative cell walls; composed of lipid A plus core polysaccharide plus O antigen (O polysaccharide side chain); released on lysis of the cell during infection. The lipid A component is responsible for endotoxin activity effects on the host; the O side chain is the antigenic portion of the LPS molecule.
- endotracheal** Within the trachea.
- engineering controls** Controls that isolate or remove a hazard from the workplace.
- enriched media** Media that contain nutritional enhancement to allow growth of fastidious microbes.
- enrichment broth** Liquid medium designed to encourage the growth of small numbers of a particular organism while suppressing other biota present.
- enteric** Member of the order Enterobacterales consisting of organisms that all ferment glucose; almost all fail to produce cytochrome oxidase; almost all reduce nitrates to nitrites. Most are resident biota of the gastrointestinal tract.
- enteric fever** Systemic bacterial infection characterized by diarrhea and prolonged fever; associated with *Salmonella* spp.
- enteritis necroticans** *Clostridium perfringens* type C food poisoning.
- enteroaggregative *Escherichia coli* (EAEC)** Pathogenic *E. coli* strains defined by their attachment to HEp-2 cells; cause persistent pediatric diarrhea.
- enterohemorrhagic *Escherichia coli* (EHEC)** *E. coli* strains producing Shiga toxins, resulting in hemorrhagic diarrhea and colitis and sometimes hemolytic uremic syndrome.
- enteroinvasive *Escherichia coli* (EIEC)** Diarrheagenic *E. coli* strains that invade the intestinal mucosa and cause dysentery similar to that caused by *Shigella* spp.
- enteropathogenic *Escherichia coli* (EPEC)** Diarrheagenic *E. coli* strains that cause severe infantile diarrhea.
- enterotoxigenic *Escherichia coli* (ETEC)** Diarrheagenic *E. coli* strains producing toxins similar to those produced by *Vibrio cholerae*.
- enterotoxin** Protein exotoxin secreted by living or lysed bacteria that alters cell function or damages membranes of the gastrointestinal tract.
- enterotoxin-mediated diarrhea** Diarrheal illness that manifests primarily as a result of the toxin produced by the infecting organism.
- entomophthoromycosis** Chronic subcutaneous form of infection caused by fungi of the order Entomophthorales (present in organic debris—*Conidiobolus coronatus*, *Conidiobolus incongruus*, and *Basidiobolus ranarum* (previously known as *Basidiobolus haptosporus*). It often occurs as the result of traumatic implantation of the organisms in the skin.
- envelope** See **enveloped virus**.
- enveloped virus** Virus surrounded by a phospholipid membrane of host cell origin.
- environmental culture** Culture taken of the environment, such as air, water, or surfaces.
- Environmental Protection Agency (EPA)** Agency of the federal government responsible for regulating environmental pollution and environmental quality.

epidemic relapsing fever Louseborne relapsing fever caused by *Borrelia* spp.

epidemiologic curve Graph plotted during a disease outbreak investigation so the number of events (cases) can be compared with the time of their development.

epididymitis Inflammation of the epididymis affecting men, usually between 19 and 35 years of age; generally a complication of chlamydia or gonorrhea.

epiglottitis Rapidly progressive infection of the epiglottis and adjacent structures usually caused by bacteria.

epitope Specific region of an antigen that binds to an antibody or T-cell receptor; also called an *antigenic determinant*.

Epstein-Barr virus (EBV) Virus that can cause infectious mononucleosis. It primarily infects B cells, where it forms a latent infection that persists for life; also called *human herpesvirus 4* (HHV-4).

erysipelas Acute spreading skin lesion that involves the subcutaneous tissues. The lesion is intensely erythematous, with a plainly demarcated but irregular edge.

erysipeloid Infection in humans caused by *Erysipelothrix rhusiopathiae*.

erythema migrans (EM) Classic target-shaped or bull's-eye skin lesion normally found at the site of a tick bite during infection by *Borrelia burgdorferi*.

erythrasma Skin disease that can result in pink patches, which can turn into brown scales.

erythrocytic phase Part of the life cycle of malaria occurring within red blood cells.

eschar Dark area where necrosis occurs in the center of a necrotic lesion; a scab; characteristically seen in patients with cutaneous anthrax.

Escherichia-Citrobacter-like organism Organism that produces a dry, pink colony with a surrounding halo of pink, precipitated bile salts.

Etest Commercial minimal inhibitory concentration (MIC) test that uses plastic strips impregnated with a gradient of antimicrobial concentrations. The strips are placed on an agar plate that has been inoculated with the test bacteria; following overnight incubation, the MIC is noted where the ellipse intersects the scale imprinted on the top of the strip.

etiologic agent Microorganism or virus causing a disease.

eugonic fermenter (EF) Gram-negative bacillus or coccobacillus that ferments glucose or other carbohydrates; grows on routine media, as compared with dysgonic fermenters, which do not grow on routine media. The designation *EF* means that it has yet to be placed into a specific genus.

eugonic oxidizer Gram-negative bacillus or coccobacillus that does not ferment glucose but can oxidize it; grows on routine media but is not yet placed into a specific genus.

eukarya See *eukaryote*.

eukaryote Organism with complex cell (cells); structures in which the genetic material is organized into a membrane-bound nucleus (nuclei), and other organelles.

eumycotic mycetoma Mycetoma caused by fungal organisms, usually comprised of wide septate hyphae.

exanthem Eruptive disease, especially one accompanied by fever, such as smallpox or measles.

exfoliative toxin Toxin that affects the epidermal layers of the skin. Also known as epidermolytic toxin associated with staphylococcal scalded skin syndrome.

exoerythrocytic phase Part of the life cycle of malaria that begins when the sporozoites are injected into humans from the mosquito. The sporozoites first invade liver cells.

exogenous Originating outside of an organism, as opposed to an endogenous agent.

exogenous anaerobe Anaerobe that exists outside of the body.

exopolysaccharide (EPS) Carbohydrate substance secreted by a number of microorganisms; used for attachment and is a major component of biofilms.

exotoxin Toxic protein produced by a bacterium and released into its environment; may exert adverse effects quite remote from the site of infection.

expectorated sputum Material from the lower respiratory tract produced by a deep cough.

exposure control plan OSHA Bloodborne Pathogen Standard safety requirement that the employer must have in place to protect the employee from bloodborne pathogens; must be annually reviewed and updated and available to all employees.

extended-care facility (ECF) Facilities in which patients are frequently immunosuppressed by disease, age, or therapy. Extended-care settings include skilled nursing facilities, nursing homes, assisted living centers, rehabilitation centers, and hospice care settings.

extended-spectrum β -lactamase (ESBL) β -Lactamase produced by *Escherichia coli*, *Klebsiella* spp., *Proteus mirabilis*, and other members of the order Enterobacterales that hydrolyze and render inactive penicillins, cephalosporins, and aztreonam.

F

facilitator Person with no vested interest in a problem whose role is to use problem-solving skills and experience to help team members resolve a problem.

facultative anaerobe Microorganism that does not require oxygen for growth but will use oxygen and grow better if it is present.

false-negative Negative test result in an individual with the disease in question.

false-positive Positive test result in an individual without the disease in question.

family In biological classification, a rank or a taxon in the rank between an order and genus.

fast-acting antiseptic Phrase used to describe an antimicrobial that exhibits the most rapid action of antiseptics; usually the rapid action of antiseptic is expressed in seconds to minutes.

fastidious Hard to grow; requires additional growth factors.

fecal leukocytes White blood cells found in stool.

fermentation Process in which a molecule is oxidized to produce energy without an exogenous electron acceptor; often used by cells when oxygen is unavailable.

fermentative Bacteria that are able to use glucose or other carbohydrates in the absence of oxygen by using an organic molecule as a final electron acceptor.

filamentous Composed of or containing filaments.

filamentous hemagglutinin Virulence factor of certain *Bordetella* spp. believed to facilitate attachment of bacteria to ciliated epithelial cells.

filariform larva Larval stage infective for the definitive host.

filtration Passing liquids through thin membrane filters composed of plastic polymers or cellulose esters containing pores of a certain size. The liquid is pulled (vacuum) or pushed (pressure) through the filter matrix. Organisms larger than the size of the pores are retained; air filtration using high-efficiency particulate air filters.

fimbria (pl. fimbriae) Nonflagellar, sticky, proteinaceous, hairlike appendage that adheres some bacterial cells to each other and to environmental surfaces.

Fitz-Hugh-Curtis syndrome Perihepatitis occurring as a complication of *Neisseria gonorrhoeae* infection; characterized by fever, upper quadrant pain, and tenderness and spasm of the abdomen.

flagella Exterior protein filaments that rotate; used by microorganisms for motility.

fluorescein isothiocyanate Fluorescent molecule commonly bound to antibody and used in diagnostic tests.

fluorescence resonance energy transfer (FRET) FRET occurs between two dye molecules held in very close proximity to each other. One dye is a donor, the other an acceptor. Two dyes may be used in conjunction to generate fluorescence, as in dual-probe FRET, or a fluorescent dye and quenching molecule may be used in conjunction to keep fluorescence low until the two molecules are separated from each other, as in the 5' nuclease assay.

fluorochrome Chemical used in fluorescent immunoassays that absorbs light of one wavelength and emits light of a different wavelength.

fluorogenic substrate Nonfluorescent molecule that becomes fluorescent when cleaved by a specific enzyme.

fluorophore Fluorescent dye molecule attached to probes used in molecular assays.

focused monitors Process created to monitor a suspected problem.

folliculitis Inflammation involving the hair follicles as a result of infection or irritation.

fomite Nonliving object that can carry or transmit infectious particles.

Food and Drug Administration (FDA), U.S. Federal agency in the U.S. Department of Health and Human Services established to regulate the release of new foods, drugs, medical devices, and other health-related products.

fungemia Fungal invasion of the bloodstream.

furuncle Painful swollen area on the skin caused by a staphylococcal infection that involves a hair follicle; also called a boil.

fusiform Spindle-shaped or tapered at each end.

G

β -galactosidase Enzyme capable of hydrolyzing lactose to glucose and galactose.

β -galactoside permease Bacterial membrane protein facilitating the uptake of β -galactosides.

gamete One of two cells, a male and a female, whose union is necessary in sexual reproduction.

gametocyte The sexual cell of Apicomplexa; the female (macrogametocyte) and male (microgametocyte) that develop into gametes.

gas gangrene Bacterial infection that produces gas within tissues; may lead to myonecrosis and sepsis; a medical emergency.

gelatinase Proteolytic enzyme that breaks down gelatin to amino acids.

genital herpes Viral infection in the genitals caused by herpes simplex virus.

genital ulcer disease (GUD) Group of sexually transmitted diseases causing ulcers on the genital area, such as syphilis and genital herpes.

genotype Genetic makeup of an organism.

genus In biological classification, a rank or a taxon in the rank between a family and species.

germ theory of disease Theory that all infectious diseases are caused by the activity of microorganisms.

germ tube Tubelike projection from a blastoconidium or spore, without a constriction at its base.

glanders Disease caused by *Burkholderia mallei*.

glycolysis Sequence of reactions that converts glucose into pyruvate, with the concomitant production of a relatively small amount of adenosine triphosphate. Glycolysis can be carried out anaerobically (in the absence of oxygen) and is thus an especially important pathway for organisms that can ferment sugars.

glycopeptide Class of antibiotic drugs that consists of glycosylated cyclic or polycyclic peptides, such as vancomycin and teicoplanin. This class of antibiotics inhibits the synthesis of cell walls in susceptible microbes by inhibiting peptidoglycan synthesis.

glycylcycline Class of antibiotic drugs derived from the tetracyclines. Similar to tetracyclines, this class of antibiotics binds to the 30S ribosomal subunit to prevent the aminoacyl tRNA from binding to the A site of the ribosome.

gonorrhea Sexually transmitted disease caused by the bacterium *Neisseria gonorrhoeae*.

gonorrheal ophthalmia neonatorum Caused by *Neisseria gonorrhoeae*, a form of conjunctivitis acquired by a newborn during birth when the infant's eyes are contaminated during passage through the birth canal of a mother infected with *N. gonorrhoeae*. *Chlamydia trachomatis* can produce a similar infection.

gram-negative bacteria Bacteria that do not retain the crystal violet complex; stained red by the safranin counterstain.

gram-negative intracellular diplococci (GNID) Gram-negative cocci in pairs found inside a phagocyte or polymorphonuclear cell.

gram-positive bacteria Bacteria that retain the crystal violet-iodine complex and appear blue on Gram-stained smears.

Gram stain Method of staining microorganisms using a violet stain, followed by an iodine solution, decolorizing with an alcohol or acetone solution, and counterstaining with safranin. The retention of the violet color of the stain or the pink color of the counterstain serves as a primary means of identifying and classifying bacteria.

granulocytopenia Disorder characterized by an abnormally low concentration of granulocytes in the blood.

granuloma An aggregate of immune cells that forms in response to a persistent inflammatory stimulus; can be contained in a fibrotic capsule.

granuloma inguinale Genital ulcerative disease caused by the intracellular gram-negative bacterium *Klebsiella granulomatis* (formerly *Calymmatobacterium granulomatis*); also called *donovanosis*.

granulomatous amebic encephalitis (GAE) An infection of the brain caused by the free-living amebae *Acanthamoeba* spp. and *Balamuthia mandrillaris*.

GRASE Term used to describe nonprescription drug products that are “generally recognized as safe and effective.”

gummas Granulomatous lesions in skin, bones, and liver; clinical manifestation of tertiary syphilis.

H

H antigen Flagellar antigen.

HACEK Specific species of the genera *Haemophilus*, *Aggregatibacter* (*Actinobacillus*), *Cardiobacterium*, *Eikenella*, and *Kingella* have been grouped together to form the acronym HACEK (first letter of each genus).

halophile Salt-loving; an organism that grows best in media or environment with an increased concentration of NaCl.

hand hygiene Refers to techniques used to ensure that hands are clean and microbe-free. Hand hygiene involves washing with soap and water if the hands are soiled or using alcohol hand rubs if the hands are not soiled.

health care antiseptic drug product Antiseptic-containing drug product applied topically to the skin to help prevent infection or prevent cross-contamination.

health care personnel hand wash Antiseptic-containing preparation designed for frequent use; reduces the number of transient microorganisms on intact skin to an initial baseline level after adequate washing, rinsing, and drying; broad-spectrum, fast-acting, and, if possible, persistent.

healthcare-associated bacteremia Bacteria in the bloodstream of patients who are hospitalized or living in a nursing home or other facility.

healthcare-associated, community-onset methicillin-resistant *Staphylococcus aureus* (HACO-MRSA) See methicillin-resistant *Staphylococcus aureus* (MRSA).

healthcare-associated infection (HAI) Infection acquired in a health care setting.

hemagglutinin Glycoprotein antigen on influenza viruses that facilitates attachment of the virus to susceptible host cells. The antigen also gets its name from its ability to clump erythrocytes.

α -hemolysin Cytolytic toxin produced by *Staphylococcus aureus* that can damage erythrocytes, platelets, and macrophages.

β -hemolysin Cytolytic toxin, also known as *sphingomyelinase C*, that acts on the sphingomyelin membrane component of erythrocytes.

hemolysis *Lysis* pertains to dissolution or breaking apart; *hemo* pertains to red blood cells. A reaction, especially caused by enzymatic or toxin activity of bacteria, observed in the medium immediately surrounding or underneath the colony.

α -hemolysis Partial lysing of erythrocytes in a blood agar plate around and under the colony; results in a green discoloration of the medium.

β -hemolysis Complete clearing of erythrocytes in a blood agar plate around or under the colonies caused by the complete lysis of red blood cells.

hemolytic-uremic syndrome (HUS) Clinical syndrome characterized by the destruction of red blood cells, damage to the lining of blood vessel walls, and in severe cases, kidney failure.

hepatitis Inflammation of the liver. Can be caused by any of the hepatitis viruses, chemicals, drugs, alcohol, or certain genetic disorders. The hepatitis viruses can be transmitted in different ways: contact with human blood, sexually, and the fecal-oral route.

heterophile antibody Antibody produced in an individual in response to one immunogen that can also bind to a different but similar molecule (antigen).

heteroploid Cells that contain an abnormal and variable number of chromosomes that is not a multiple of the normal haploid number.

heteroresistant Population of cells in which some cells appear susceptible and others appear resistant.

heterotroph Organism that requires organic substrates as a source of carbon for metabolism.

hexacanth embryo (oncosphere) First larval stage of most tapeworms found within the egg, characterized by the presence of six hooklets.

hidradenitis suppurativa Skin disease that affects areas that maintain apocrine sweat glands and hair follicles, such as the axillae, groin, and buttocks and under the breasts of women.

high-level aminoglycoside resistance In enterococci, indicates resistance to high concentrations of aminoglycosides (e.g., gentamicin or streptomycin) that usually result from production of aminoglycoside-modifying enzymes. Enterococci with high-level gentamicin resistance do not show synergism with ampicillin, penicillin, or vancomycin; streptomycin performs similarly.

highly active antiretroviral therapy (HAART) Therapy that, when properly prescribed and used, can improve the quality of life and longevity of those infected with human immunodeficiency virus, although it does not cure the infection.

hippurate hydrolysis Test used for the presumptive identification of group B streptococci (*Streptococcus agalactiae*). The hippuricase enzyme found in group B streptococci hydrolyzes sodium hippurate to sodium benzoate and glycine.

Hodgkin disease (HD) Type of lymphoma characterized by swollen lymph nodes and the presence of large malignant cells called *Reed-Sternberg cells*, which are multinucleated or have a bilobed nucleus (resembling an owl's eye) with prominent eosinophilic inclusion-like nucleoli.

homogenization Preparation of tissue for microbiology culture by grinding to release microbes from cells and produce an even suspension.

hospital-associated methicillin-resistant *Staphylococcus aureus* (HA-MRSA) See methicillin-resistant *Staphylococcus aureus* (MRSA).

housekeeping gene Gene that codes for basic functional proteins for cells. They are always expressed. They are good targets for internal controls from clinical specimens.

human blood bilayer Tween (HBT) agar Medium of choice for the isolation and presumptive identification of *Gardnerella vaginalis*.

human granulocytic anaplasmosis (HGA) Infectious disease caused by *Anaplasma phagocytophilum* (formerly known as *Ehrlichia phagocytophilum*). The symptoms closely resemble those of human monocytic ehrlichiosis. See **human monocytic ehrlichiosis**.

human immunodeficiency virus (HIV) Retrovirus that infects components of the human immune system, primarily CD4⁺ T cells, macrophages, and dendritic cells.

human monocytic ehrlichiosis (HME) Disease caused by *Ehrlichia chaffeensis*.

human papillomavirus (HPV) A virus that causes the most common sexually transmitted viral disease in the United States, genital warts. Certain genotypes cause various forms of cancer in the genital area, particularly in the cervix.

humoral immune response See **humoral-mediated immunity**.

humoral-mediated immunity Type of immune response that involves circulating antibodies.

hyaluronidase Enzyme, also referred to as *spreading factor*, that solubilizes the ground substance of mammalian connective tissues (hyaluronic acid).

hybrid capture Signal amplification method. An RNA probe is annealed to target DNA; a capture antibody binds the DNA-RNA hybrid to a solid surface. A probe labeled with alkaline phosphatase (AP) then anneals to the hybrid, and the AP substrate is added to the system; cleavage of the substrate results in light emission, detected by a luminometer.

hybridoma Cell formed from the fusion of a normal B cell to a myeloma cell, a cancerous plasma cell, so the resulting cell is able to secrete monoclonal antibodies and is immortal.

hydroxyl radical (OH⁻) An intermediate reactive generated when the superoxide anion reacts with hydrogen peroxide in the presence of iron (Fe³⁺/Fe²⁺). Short-lived molecule that is the most potent biological oxidant known.

hypha (pl. hyphae) Long strand of fungal cells, with or without cross walls; filaments interweave to form mats called *mycelia*.

I

iatrogenic Pertains to an event caused by a medical intervention.

iatrogenic infection Infectious condition caused by a medical intervention.

immune complex Combination of antigen and antibody bound together.

immune response Resultant action of the immune system, such as antibody production to an immunogen; response of the immune system to an antigen.

immune system Organ system that protects an organism from outside biological influences.

immunocompetent Term used to describe the ability of an immune system to mobilize and deploy its antibodies and other responses to stimulation by an immunogen.

immunocompromised Term used to describe an individual with deficient function of the immune system.

immunocompromised hosts See **immunocompromised**.

immunodiffusion Movement of antigens or antibodies through a matrix, such as agarose.

immunogen Molecules capable of stimulating an immune response.

immunoglobulin Complex group of serum proteins produced by B cells and plasma cells in response to foreign immunogens. In humans there are five isotypes: IgG, IgM, IgA, IgD, and IgE.

immunoglobulin A (IgA) Antibody defined by the α heavy chain. This is the immunoglobulin found in greatest concentration in secretions.

immunoglobulin D (IgD) Antibody defined by the δ heavy chain.

immunoglobulin E (IgE) Antibody defined by the ϵ heavy chain. IgE is primarily attached to mast cells and serves as the antigen recognition for type I hypersensitivity reactions to allergens and immunity to parasites.

immunoglobulin G (IgG) Antibody defined by the γ heavy chain. This is the primary immunoglobulin found in human serum.

immunoglobulin M (IgM) Antibody defined by the μ heavy chain. Found in human serum as a pentamer, but monomers are embedded into mature B-cell membranes.

immunosenescence Naïve T cells are no longer available to be stimulated by immunogens, which leads to the decline of the immune response over time.

immunosuppression Term used to describe the state of an immune system that is suppressed.

impetigo Localized skin disease that begins as small vesicles and progresses to weeping lesions.

in situ amplification (ISA) Technique that couples polymerase chain reaction amplification with in situ hybridization. The method is used to amplify DNA directly from tissue specimens, intact cells, or chromosomal material; used to increase the sensitivity and specificity of in situ hybridization reactions.

in situ hybridization Hybridization method in which target DNA or RNA is detected directly in intact cells, tissue, or chromosomal material with a labeled probe.

incidence Number of new cases of a disease over a time period (e.g., weeks, months, years).

index case First case in an outbreak investigation.

indifference Occurs when the antimicrobial activity of a combination of antimicrobial agents is equal to the activity of the individual agents.

indigenous microbiota Microorganisms of low virulence normally found in or on body sites; synonymous with *usual microbiota*.

indirect agglutination Agglutination assay in which an antigen is artificially attached to a carrier particle, such as latex beads.

indirect fluorescent antibody test Immunoassay using a known antigen affixed to a microscope slide that binds antibody in patient's serum. A second fluorochrome-labeled, antihuman antibody is then added.

indirect sandwich immunoassay Immunoassay using a known antigen affixed to a solid surface that binds antibody in patient's serum. A second labeled antihuman antibody is then added.

individualized quality control plan (IQCP) An all-inclusive approach to assuring quality. It includes many practices that a laboratory uses to ensure quality testing. An IQCP must address the potential failures and errors identified in the entire testing process: preanalytic, analytic, and postanalytic phases of testing.

indole test Bacteriologic test to determine an organism's ability to form indole from tryptophan with the enzyme tryptophanase.

induced resistance Resistance to a compound caused by induction by the antimicrobial agent itself, resulting in differential resistance gene expression.

induced sputum Material from the lower respiratory tract collected following aerosol induction.

infection control risk assessment (ICRA) Review of a situation, such as new construction, to determine any infection control risks that may be present.

infection prevention and control (IPC) A practical, evidence-based approach to prevent patients and health care workers from being harmed by avoidable infection.

infection prevention and control practitioner (IPCP) Individual in a health care setting whose responsibility is to institute outbreak investigations, develop procedures to prevent the acquisition of infections, and implement infection control practices.

infection preventionist See **infection prevention and control practitioner**.

infection rate Occurrence of infections within a given at-risk population, expressed as a percentage or as a rate.

inflammation Physiologic reaction of vascularized tissue to injury involving physical symptoms of pain, redness, swelling, and tenderness caused by accumulation of plasma and white blood cells; part of innate immunity.

inhalation anthrax Form of anthrax that results from inhaling anthrax spores. This is the most likely form of anthrax that would result from a release of anthrax spores into the air.

innate immune response See **innate immunity**.

innate immune system See **innate immunity**.

innate immunity Natural form of host protection; nonspecific and not stimulated by specific antigenic stimuli.

insertion sequence (IS) Mobile genetic elements known to encode only functions involved in insertion events.

integron Mobile DNA element that can capture and carry genes, especially those that carry antimicrobial drug resistance.

interferon (IFN) Class of mediators that increases the resistance of cells to viral infection by inhibiting viral replication and the growth of some euplastic cells.

interleukin (IL) Type of cytokine originally thought to be produced by and to act on leukocytes; however, it has since been discovered that other types of cells can elaborate some interleukins and that interleukins also have effects on cells other than leukocytes.

intermediate Refers to the interpretation of antimicrobial susceptibility test results implying that the agent might be effective for infections located at body sites where the drugs are physiologically concentrated, or when a high dosage of drug can be used. The intermediate category also includes a buffer zone,

which should prevent small, uncontrolled technical factors from causing major discrepancies in interpretation.

intermediate host Individual in which a parasite has its larval and or asexual reproductive stage.

intermittent bacteremia When organisms are periodically released from the primary site of infection into the bloodstream. This occurs as the result of abscesses or as a clinical manifestation of certain types of infections.

intertrigo Yeast infection of skin folds caused by *Candida albicans*.

intervention Implementation of procedures to stop the spread of an infection or outbreak.

intravascular device Device such as a catheter that is inserted into a blood vessel.

intrinsic resistance Type of antimicrobial resistance; an inherent genotypic characteristic disseminated horizontally to progeny. Mechanisms that mediate intrinsic resistance to antimicrobials include cell wall impermeability, efflux, biofilm formation, and expression of genes mediating inactivating enzymes.

isolation streak Technique to spread inoculum over the surface of agar plates so individual colonies can be obtained and semi-quantitative analysis performed.

J

Jarisch-Herxheimer reaction Systemic reaction of fever, chills, headache, myalgias, and exacerbation of cutaneous lesions believed to be caused by the rapid release of endotoxin from organisms shortly after initiation of antimicrobial therapy.

JEMBEC (James E. Martin Biological Environmental Chamber) system Commercial system for collection of specimens for *Neisseria gonorrhoeae*; contains selective agar and a CO₂-generating tablet.

K

K antigen Capsular surface antigen possessed by some strains of Enterobacterales, particularly *Salmonella enterica* serotype Typhi, in which it is called the *Vi antigen*.

Kanagawa phenomenon Refers to heat-stable hemolysin production by most strains of *Vibrio parahaemolyticus*, able to lyse human red blood cells in a special, high-salt, mannitol medium (Wagatsuma agar). Production of β -hemolysis on this agar is called the *Kanagawa phenomenon*.

karyosome Spherical chromatin mass within the nucleus of protozoa.

keratitis Inflammation of the surface or connective tissues of the cornea.

keratoconjunctivitis Inflammation of the ocular external surfaces (conjunctiva and cornea epithelia).

ketolide Semi-synthetic derivative of 14-member ring macrolides; has a carbonyl group at the C3 position, which is crucial in conferring sensitivity to macrolide-resistant strains. Ketolides share many of the characteristics of the advanced macrolides.

kinetoplast Structure found in some blood and tissue protozoans composed of a granule, from which the flagellum or undulating membrane arises, and a mitochondrion.

Kinyoun stain Acid-fast stain used to visualize mycobacteria and certain coccidian parasites. Like the Ziehl-Neelson stain, the Kinyoun stain is a carbolfuchsin method; however, it does not involve heat application.

Kirby-Bauer test Type of antimicrobial susceptibility test in which filter paper disks impregnated with antimicrobial agents are placed on the surface of an agar plate that has been inoculated with the test bacteria. Following overnight incubation, zones of inhibition of growth are measured; results for each agent tested are interpreted as susceptible, intermediate, or resistant based on predefined Clinical and Laboratory Standards Institute criteria; also referred to as *antimicrobial disk diffusion test*.

Klebsiella-Enterobacter-like organisms Organisms that produce large, mucoid, pink colonies; occasionally have cream-colored centers.

Kligler iron agar (KIA) Bacteriologic medium that aids in determining carbohydrate fermentation (glucose and lactose) and H₂S formation.

Koplik spots White spots that appear on the mucous membranes of measles patients approximately 1 day before the appearance of the typical measles rash.

Krebs cycle A series of enzyme catalyzed reactions that reduce the acetyl portion of acetyl coenzyme A; also referred to as the *tricarboxylic acid cycle*.

L

L form Bacterium that has temporarily lost its cell wall as a result of environmental conditions.

laboratory-acquired infection (LAI) An infection occurring as a result of laboratory-related activities.

Laboratory Response Network—Biological

(LRN-B) National program for detection and identification of biothreat agents.

β-lactam antibiotic Antibiotic that contains the β-lactam ring, the structure essential for the antibacterial activity of the antibiotic in this class.

β-lactam ring Structure found in penicillins, cephalosporins, monobactams, and carbapenems. This four-member ring functions as a structural analogue of the normal substrate acyl-D-alanyl-D-alanine.

β-lactamase Enzyme produced by bacteria that destroys the activity of β-lactam agents by hydrolyzing the β-lactam ring portion of the β-lactam molecule; many types of β-lactamase affect specific β-lactam agents.

β-lactamase inhibitor (BLI) Molecule that inactivates the enzyme activity of β-lactamases preventing them from destroying the β-lactam ring found in some antibiotics.

lactoferrin Multifunctional protein with antimicrobial activity that is one of the innate defense proteins found mainly in mucosal secretions such as tears. This protein is present in secondary granules of polymorphonuclear neutrophils. It belongs to the transferrin family of proteins showing a high affinity for iron (ferric state).

lactose fermenters Bacteria able to metabolize lactose without oxygen typically forming acid end products. Easily detected by the color change they produce on media; as the pH changes when lactose is fermented, the organisms produce colored colonies, depending on the pH indicator. Colonies of nonfermenters remain clear and colorless.

Lancefield classification Classification of streptococci based on a surface antigen, the C carbohydrate.

latent phase In syphilis, the period in late syphilis when it passes into a silent period (asymptomatic), which may last for many years.

Lean A quality improvement tool to minimize waste and at the same time maximize customer value.

Learn from Defect A process quality improvement tool to evaluate risks; provides a methodical and structured approach to ensure that every component of the process is assessed.

Legionnaires' disease Febrile disease with pneumonia (atypical) caused by *Legionella* organisms.

leprosy Infection of the skin, mucous membranes, and peripheral nerves caused by *Mycobacterium leprae*.

leptospirosis Zoonotic disease in humans caused by *Leptospira interrogans*.

lethal factor One of three proteins that make up the anthrax toxin. The combination of lethal factor with protective antigen results in cell death.

leucine aminopeptidase (LAP) Enzyme used in the test for presumptive identification of streptococci. It is a peptidase that hydrolyzes peptide bonds adjacent to a free amino group; called *LAP* because it reacts most quickly with leucine.

leukocidin Toxin secreted by certain bacterial species toxic to leukocytes (e.g., Panton-Valentine leukocidin produced by *Staphylococcus aureus*).

linezolid Antibiotic thought to bind to the 50S ribosomal subunit of prokaryotes, preventing formation of the preinitiation complex, with the 30S ribosomal subunit containing bound initiation factors.

lipooligosaccharide (LOS) Glycolipid consisting of lipid A and core structures similar to lipopolysaccharide. LOS is found in the cell wall of some gram-negative bacteria including *Neisseria*, *Campylobacter*, and *Haemophilus* species.

lobomycosis Chronic fungal infection of the skin caused by *Lacazia loboi* (previously known as *Loboa loboi*); mainly found in the tropics of South America and Central America. The organisms are thought to reside in soil or vegetation and infect humans via skin trauma.

Loeffler medium (Loeffler agar) Nonselective medium containing serum; generally used for growing *Corynebacterium* spp.

Löwenstein-Jensen medium Egg-based, opaque, solid medium commonly used in clinical laboratories for growth of mycobacteria.

Lyme borreliosis Systemic, multistage illness (Lyme disease) caused by *Borrelia burgdorferi* infection following a tick bite.

lymphocyte Type of white blood cell that develops in the bone marrow but matures in other areas such as the thymus, spleen, and lymph nodes.

lymphogranuloma venereum (LGV) Severe, invasive, sexually transmitted disease associated with specific serovars (L1, L2, L2a, L3) of *Chlamydia trachomatis*.

lymphokine Chemical messenger produced by lymphocytes that carry messages among the cells of the immune system. Examples include interferon, which initiates defensive

reactions to viruses, and the interleukins, which activate specific immune cells.

lysine iron agar (LIA) Bacteriologic medium determining lysine metabolism and the ability to produce H_2S .

lysogeny Incorporation of the genetic material of a bacteriophage with that of the host bacterium.

lysozyme Enzyme that destroys bacterial cell walls by hydrolyzing the polysaccharide component of the cell wall. Lysozyme can be found in the mucosal membranes that line the human nasal cavity and tear ducts.

M

M protein Antigen found in the cell wall of *Streptococcus pyogenes* that is important for virulence (attachment) and is not found in the other Lancefield group streptococci.

macroconidium Larger of two types of conidia produced by fungi, typically multicelled.

macrolide Class of antibiotic drugs, such as erythromycin, clarithromycin, and azithromycin, that targets the 50S subunit specifically by binding to the peptidyltransferase cavity in the proximity of the A and P loops, near adenine 2058 of 23S rRNA.

macroscopic observation Gross appearance or physical characteristics of the specimen.

major outer membrane protein (MOMP) Predominant protein present in the outer membrane of bacteria. These proteins often exhibit antigenic variation, allowing serogrouping of bacteria, such as with *Chlamydia trachomatis*.

margin Border, or edge.

material safety data sheet (MSDS) Information provided by the manufacturer or distributor for hazardous chemicals.

matrix-assisted laser desorption/ionization–time-of-flight (MALDI-TOF) mass spectrometry Mass spectrometry approach that includes the use of molecular and mass spectrometry methods to examine protein profiles; expected to increase the ability to identify clinically relevant microorganisms accurately and rapidly.

Maurer dots Coarse granulations present in red blood cells invaded by the parasite *Plasmodium falciparum*.

McFarland turbidity standards Suspensions of latex particles or barium sulfate prepared at various densities to represent specific numbers of bacteria. For example, the turbidity of a McFarland 0.5 standard is comparable to the turbidity of a bacterial suspension containing 1.5×10^8 colony-forming units/mL.

mecA Gene that codes for penicillin-binding protein 2a, which confers oxacillin resistance in staphylococci.

median infectious dose (ID_{50}) Average amount or number of organisms that will cause an infection in 50% of those who acquire the organism.

mediastinitis Clinical condition characterized by widening of the mediastinum, the central compartment of the thoracic cavity. Although not specific for anthrax, it is a common radiologic finding in patients with inhalation anthrax.

medusa head Term used to describe the colony appearance of *Bacillus anthracis*.

melioidosis Disease caused by *Burkholderia pseudomallei*.

melting curve analysis Real-time polymerase chain reaction (PCR) method used to determine whether nonspecific PCR products or primer-dimers have formed; also used to

determine the identity of a target. Fluorescence is observed in real-time PCR when a fluorophore is annealed to a PCR product. When the temperature of an assay is increased for melting curve analysis, the fluorophore will be released from the PCR product by denaturation. This causes a sudden decrease in fluorescence, which can be read as a peak—the temperature at which a fluorophore is released from the target sequence.

melting temperature (T_m) Temperature at which a double-stranded nucleic acid molecule is 50% hybridized and 50% dissociated. A temperature below the T_m is often used for hybridization reactions, including the polymerase chain reaction.

meningismus Clinical manifestation characterized by nuchal rigidity, headache, and photophobia, without the presence of inflammation or infection but seen in concordance with other acute illnesses in children.

meningitis (leptomeningitis) Inflammation of the meninges, the protective membrane covering the brain and spinal cord. Although the swelling may be caused by a bacterial or viral infection of the membrane that surrounds the brain and spinal cord, injuries, cancer, and other types of infections may also cause meningitis.

meningococcemia Presence of meningococci (*Neisseria meningitidis*) in the bloodstream.

meningoencephalitis Presence of inflammation in the meninges and the brain.

merozoite Parasitic form produced during schizogony. In malaria, these structures are released from a ruptured erythrocyte and invade other erythrocytes at the end of an asexual reproductive cycle. Some merozoites differentiate into gametocytes.

mesophile Organism that grows best in moderate temperatures, 25° to 40° C.

metacercaria For most flukes, this is the life-cycle stage infective for humans. It develops after the cercaria invades the tissue of the second intermediate host or contacts water or vegetation, loses its tail, and develops a protective wall.

metagenomics Study (genomic analysis) of the genetic material of all microorganisms recovered directly from environmental samples using molecular techniques.

methicillin-resistant *Staphylococcus aureus* (MRSA) Strain of *S. aureus* that contains *mecA* and PBP2a and is resistant to methicillin and other β -lactamase-resistant penicillins such as oxacillin or nafcillin.

methicillin-resistant *Staphylococcus epidermidis* (MRSE) Strain of *S. epidermidis* that is resistant to methicillin and other β -lactamase-resistant penicillins such as oxacillin or nafcillin.

methicillin-sensitive *Staphylococcus aureus* (MSSA) Strain of *S. aureus* that is easier to treat than MRSA because it is susceptible to methicillin and other β -lactamase-resistant penicillins such as oxacillin or nafcillin.

methicillin-susceptible *Staphylococcus aureus* (MSSA) Strain of *Staphylococcus aureus* susceptible to methicillin and other β -lactamase-resistant penicillins such as oxacillin or nafcillin.

methyl red–Voges-Proskauer (MRVP) test Bacteriologic test determining the end products of glucose fermentation: methyl red test, mixed acids; Voges-Proskauer test, stable neutral products.

- microaerophile** Microorganism that grows in conditions of reduced oxygen and increased carbon dioxide.
- microaerophilic** Term used to describe microorganisms that require environments containing a concentration of oxygen lower than that present in the atmosphere (about 20%).
- microbial load** Total number of organisms present (bioburden).
- microbial morphology (morphotypes)** Description of microorganisms, their size, shape, internal structure and other characteristic features that help recognize them. Gram-positive cocci in pairs, tetrads, and grapelike clusters are a staphylococcal morphotype.
- microbiota** Microscopic organisms of a region (e.g., respiratory, urinary, gastrointestinal tract) harbored by normal, healthy individuals.
- microconidium** Smaller of two types of conidia produced by fungi; usually single-celled, rarely two-celled.
- microfilaria** Larval form of filarial worms.
- Middlebrook 7H10 and 7H11 agars** Serum albumin-based clear agar media used to support the growth of mycobacteria.
- miliary tuberculosis** Disseminated disease due to the hematogenous spread of tubercle bacilli, often fatal.
- minimal bactericidal concentration (MBC)** Lowest concentration of antimicrobial agent that kills 99.9% of the test bacteria.
- minimal inhibitory concentration (MIC)** Lowest concentration of antimicrobial agent that inhibits the growth of a bacterium.
- minimal medium** Laboratory growth medium whose contents are simple and defined; not generally used in the diagnostic microbiology laboratory.
- miracidium (pl. miracidia)** First-stage, ciliated, free-swimming larva of flukes that emerges from the egg and penetrates a specific species of snail to continue the fluke life cycle.
- Moeller decarboxylase base medium** Classic bacteriologic medium used to detect decarboxylase activity.
- moist heat** High temperature used with water to kill microorganisms. Heat under steam pressure; e.g., autoclaves.
- mold** Fungus characterized by hyphae; also known as *filamentous fungi*.
- molecular beacons** Real-time polymerase chain reaction (PCR) detection technique. A molecular beacon probe is a hairpin loop structure, with a fluorophore on the 5' end and a quencher on the 3' end. During the denaturation step of PCR, the molecular beacon probe dissociates, as does formed PCR product. The probe anneals to the PCR product; when this occurs, the fluorophore and quencher are no longer in close proximity. Fluorescence thus increases as the PCR product accumulates.
- molecular epidemiology** The analysis of molecules, such as proteins and nucleic acids, for the detection, identification, and characterization of microorganisms to generate isolate-specific markers to assess epidemiologic relatedness.
- molluscum contagiosum** Common skin disease caused by a poxvirus (molluscum contagiosum virus); characterized by small, firm, waxy papules, often with umbilicated centers; occasionally giant lesions may be seen.
- monoclonal antibody** Antibody solution with specificity to a single epitope.
- monograph** Developed for therapeutic classes of ingredients generally recognized as safe and effective. A manufacturer wanting to market a product containing an ingredient covered under the over-the-counter monograph need not seek the U.S. Food and Drug Administration's prior approval.
- monomicrobial** One microbe morphotype is present; if it is the likely pathogen in an infection, the infection is monomicrobial.
- morula (pl. morulae)** Intracellular inclusion suggestive of infection with *Ehrlichia* spp.
- motility-indole-ornithine (MIO) medium** Bacteriologic medium determining motility, the ability to form indole from tryptophan, and ornithine metabolism.
- mRNA translation** Messenger RNA that encodes and carries information from DNA during transcription to sites of protein synthesis (ribosomes) to undergo translation to yield a gene product. Translation is a step in the process of protein biosynthesis.
- multilocus enzyme electrophoresis (MLEE)** Nonamplified strain-typing method. Proteins are isolated from the strain of interest and separated in a gel. A probe is then used to detect a specific protein. Differences in protein migration patterns are mutations; different strains can be identified this way.
- multilocus sequence typing (MLST)** Strain-typing method that uses polymerase chain reaction to amplify several different genetic loci. The resulting fragments are separated by electrophoresis and analyzed. The same strain will have the same pattern.
- multilocus variable-number of tandem repeat analysis (MLVA)** Typing method that takes advantage of repetitive DNA sequences in genomes. MLVA amplifies regions of DNA that contain repeats. When there are different numbers of repeats at a given locus, it is referred to as a variable number of tandem repeats (VNTR). MLVA maps VNTRs among bacterial strains by using the polymerase chain reaction assay.
- multiplex polymerase chain reaction (PCR)** PCR method that uses two primer sets in the same tube. Each primer set is specific for a different target.
- mutualism** A symbiotic relationship when both species benefit from the association.
- mycelia** Intertwined hyphae that form a mat on the surface of a fungal colony; aerial mycelia grow upward away from the surface.
- mycetoma** Granulomatous subcutaneous abscesses caused by either filamentous bacteria (actinomycetoma) or fungi (eumycetoma) found in the soil and enter the body through a break in the skin.
- Mycobacterium avium complex (MAC)** Consists of *Mycobacterium avium* and *Mycobacterium intracellulare*; previously the most common systemic bacterial infection in patients with acquired immunodeficiency syndrome (AIDS) and recently has been associated with pediatric lymphadenitis.
- Mycobacterium tuberculosis complex (MTBC)** Organisms that cause the disease known as *tuberculosis*. Eleven closely related organisms are grouped together to form the *Mycobacterium tuberculosis* complex that includes *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. canettii*, and *M. microti*.

mycolic acids Complex branched-chain fatty acids with large numbers of carbon atoms; components of the *Mycobacterium tuberculosis* cell wall.

mycosis (pl. mycoses) Disease caused by fungi.

myonecrosis Necrosis of the muscle. Synonymous with *gangrene*.

N

nanobiotechnology (nanotechnology) Use of nanotechnology (sometimes called *nanobiotechnology* when used for biological purposes) in molecular diagnostics; also called *nanomolecular diagnostics*. Although there are different definitions of nanotechnology, it is useful to consider it as a technology that uses atomic and/or molecular properties to build structures starting at the molecular level, at the nanometer scale (a nanometer is a billionth of a meter).

narrow spectrum In antimicrobial activity, a limited range of activity of an antimicrobial agent.

National Fire Protection Association (NFPA) hazard rating diamond The NFPA 704 system uses a diamond-shaped diagram of symbols and numbers to indicate the degree of hazard associated with a particular chemical or material. These diamond-shaped symbols are placed on containers of chemicals or materials to identify the degree of hazard associated with the chemical or material.

National Nosocomial Infection Surveillance System (NNISS) Federal system whose goal is to monitor the incidence of healthcare-associated (nosocomial) infections and their associated risk factors and pathogens at the national level, correlating data from multiple health care settings across the United States.

necrotizing fasciitis (NF) Rapidly progressing and frequently life-threatening soft tissue infection; an infectious gangrene typically caused by several different types of bacteria such as group A streptococci.

negative predictive value (NPV) See *predictive value*.

nested polymerase chain reaction (nested PCR) Sensitive PCR technique that uses two PCR assays. The first assay produces PCR amplicon that is then used as template in the second PCR assay.

neutropenia Abnormally reduced concentration or number of neutrophils in the bloodstream.

new drug application (NDA) A request from a drug sponsor to the U.S. Food and Drug Administration (FDA) for approval to market a new drug. The NDA includes information for the FDA to determine if the drug is proven safe and effective for human use before being marketed.

nitrate reduction test Bacteriologic test determining an organism's ability to form nitrite or nitrogen gas by reducing nitrate.

o-nitrophenyl- β -D-galactopyranoside (ONPG) Chromogenic substrate that is initially colorless but is cleaved by β -galactosidase, producing the yellow compound o-nitrophenyl; this test helps to determine if an isolate is a true nonlactose fermenter.

nodular lymphangitis Disorder characterized by inflammatory nodules that occur along lymphatic vessels that drain an area of primary skin infection (also called *lymphocutaneous syndrome*).

nomenclature Devising or choosing names for organisms.

nonchromogenic Refers to *Mycobacterium* spp., such as *M. tuberculosis*, that do not produce pigmentation.

nonencapsulated Strains of bacteria and yeast that do not produce a capsule.

nonfermentative Bacteria that are unable to use glucose or other carbohydrates in the absence of oxygen.

nongonococcal urethritis (NGU) Infectious condition of the urethra in males that is not caused by gonorrheal infection.

nonlactose fermenter (NLF) See *lactose fermenters*.

nonoxidizers Bacteria that neither oxidize nor ferment glucose or other carbohydrates. They derive their energy from carbon compounds other than carbohydrates.

nonphotochromogenic Refers to characteristic appearance of some *Mycobacterium* spp. in which exposure to light does not stimulate pigment production.

nonporous surface Smooth, unpainted solid surface that limits penetration of liquid.

nonselective media Media that support the growth of most nonfastidious aerobes.

nonsusceptible Reporting category used when there are no "intermediate" or "resistant" interpretive criteria, only a "susceptible" interpretive criterion, and the minimal inhibitory concentration or disk diffusion zone size for classifying the organism as susceptible is not achieved.

nontreponemal antibody tests Tests for syphilis that detect antibodies to nontreponemal antigens. Generally, these tests are used to screen for diseases, which are then confirmed using treponemal antibody tests.

nontuberculous mycobacteria (NTM) Mycobacteria not associated with tuberculosis; some are nonpathogenic, whereas others have been commonly implicated as opportunistic pathogens. They are ubiquitous in the environment.

nontypable *Haemophilus influenzae* (NTHi) Strains of *H. influenzae* that are not encapsulated.

normal biota Microbes normally present in an organ or region of the body; they exist in a symbiotic relationship with the host. May act to help prevent infection. These organisms are isolated from the host in the absence of disease.

Northern blot Procedure that detects RNA with a specific, labeled probe. The RNA is separated by electrophoresis and then transferred to a solid membrane. The probe is labeled and detected by a variety of methods.

nosocomial Of or being a secondary disorder associated with being treated in a hospital but unrelated to the patient's primary condition.

nosocomial bacteremia See *healthcare-associated bacteremia*.

nosocomial infection Infection acquired within 72 hours of a stay in a health care facility.

novobiocin susceptibility Test useful for differentiating novobiocin-resistant *Staphylococcus saprophyticus* from other coagulase-negative staphylococci (sensitive).

nucleic acid hybridization Ability of two single-stranded nucleic acid species to anneal, or bind, to each other and form

a hybrid, or duplex. Nucleic acid molecules hybridize when they are complementary to each other.

nucleic acid sequence-based amplification

(NASBA) Amplification assay that occurs at one temperature that produces transcript from a target nucleic acid. This method uses a pair of primers and reverse transcriptase, RNase H, and T7 RNA polymerase to synthesize many transcript copies from each target nucleic acid sequence.

nucleocapsid Genome of a virus enclosed in a protein coat (the capsid).

nucleoside reverse transcriptase inhibitors (NRTIs) A class of antiviral drugs interfering with the function of the enzyme reverse transcriptase, used to treat retroviral infections.

numeric codes Number generated by a series of biochemical tests in a commercial identification kit that can be compared with numbers in a database to identify an organism.

nutrient media Culture media that are complex; made of extracts of meat or soybeans.

O

O antigen Heat-stable somatic cell antigens of the Enterobacteriaceae, notably identified in *Escherichia coli* and *Salmonella* spp.

obligate aerobe Microorganism that requires oxygen for growth.

obligate anaerobe Microorganism that can live and reproduce only in a strict anaerobic environment (0% oxygen).

obligate intracellular parasite Microorganism or virus unable to replicate independently outside a living cell.

occult (unsuspected) bacteremia Bacteremia not associated with any physical signs or symptoms of severe infection.

O/F (oxidative/fermentative) basal medium Bacteriologic medium used in determining the ability of bacteria to use carbohydrates oxidatively or fermentatively.

Ohara disease Another name for tularemia, which is caused by *Francisella tularensis*.

oligonucleotide Small nucleic acid molecule used as a primer or probe in molecular diagnostic techniques; synthetic, single-stranded, nucleic acids complementary to target sequences.

onychomycosis Infection caused by fungi involving the nails.

opaque Not transparent or translucent; impenetrable to light; not allowing light to pass through.

ophthalmia neonatorum Severe eye infection in infants caused by *Neisseria gonorrhoeae*, acquired during natural (vaginal) delivery.

opportunistic Microorganisms that usually do not produce disease but are capable of causing disease in an individual whose immune system is compromised.

opportunistic Taking advantage of a situation, exploiting weaknesses.

opportunistic infection Disease caused by a microorganism with low virulence that becomes pathogenic in a host with low immunologic resistance.

opportunistic pathogen See **opportunistic**.

opsonin Host molecule (e.g., certain complement proteins and antibodies) that enhances phagocytosis.

opsonization Process whereby microorganisms are changed so they are more easily and readily engulfed by phagocytes and macrophages.

optochin test Test that determines an organism's susceptibility to optochin (ethylhydrocupreine); used to differentiate *Streptococcus pneumoniae* (susceptible) from other α -hemolytic streptococci (resistant).

orbital cellulitis Infection of the orbital tissues.

orf Contagious viral skin disease acquired from infected sheep and goats; characterized by painless vesicles that may progress to red weeping nodules and finally to crusting and healing; caused by the orf virus in the genus *Parapoxvirus*.

organ culture Maintenance or growth of organ fragments.

orthostatic changes Changes that occur while in an erect posture or position.

ORYX The Joint Commission initiative requiring health care organizations to submit performance measurements to be analyzed and compared with those of similar institutions.

osteomyelitis Chronic or acute infection of the bone or bone structures as a result of an infective process.

otitis media Disease that results from infection of the middle ear; characterized by the presence of middle ear inflammation and fluid.

outbreak Occurrence of events, such as infections, that exceeds the normal and expected numbers.

outbreak investigation Epidemiologic investigation of an outbreak.

outcome monitors Measurements of the result of a process.

over-the-counter (OTC) drugs Nonprescription drugs that are considered to be safe and effective for consumers to use without professional supervision, provided the required label directions and warnings are followed.

oxacillin-salt screen plate Agar plate containing 6 $\mu\text{g/mL}$ of oxacillin and 4% NaCl used for detecting oxacillin-resistant *Staphylococcus aureus*.

oxazolidinone Class of azoles with the carbon between the nitrogen and oxygen oxidized to a ketone.

oxidase test Bacteriologic test to determine the presence of the intracellular enzyme cytochrome oxidase, part of the electron transport system.

oxidation Chemical process whereby electrons are donated. In bacteria, oxygen is frequently used as the terminal electron acceptor during glucose oxidation; however, other inorganic molecules can also be used.

oxidizers Bacteria that require molecular oxygen as the final electron acceptor; glucose or carbohydrates are metabolized only in the presence of oxygen.

P

pandemic An epidemic that has spread over several countries or continents, usually affecting a large number of people.

Panton-Valentine leukocidin (PVL) Staphylococcal cytolytic toxin that can act on polymorphonuclear leukocytes.

paradoxical (eagle) effect Related to minimal bactericidal concentration testing; decreased bactericidal activity of an

antimicrobial agent at higher concentrations of antimicrobial agent, as demonstrated by more colonies growing on subcultures at higher concentrations than at lower concentrations.

paraffin bait technique Assay used to recover *Nocardia* spp. and aerobic actinomycetes from contaminated samples; based on the principle that these organisms can use simple carbon sources for nutrition.

parapneumonic effusion (PPE) The filling of the pleural cavity with exudative pleural fluids, often secondary to pneumonia.

parasite Organism that lives in or on and takes its nourishment from another organism (host), while harming that organism. Parasitic diseases include infections by protozoa, helminths, and arthropods.

parasitism Relationship between species (host and organism) in which one of the species (parasite) benefits at the expense of the other (host).

paronychia Inflammation of the folds of the skin bordering the nail beds.

paroxysmal phase Second phase of pertussis characterized by the sudden onset of severe, repetitive coughing followed by a "whoop" as the individual gasps for air, sometimes followed by vomiting.

pasteurization Method of disinfection used mostly in the food industry; eliminates foodborne pathogens and organisms responsible for food spoilage. It is carried out at 72° C for 15 seconds. The main advantage of pasteurization is that treatment at this temperature reduces spoilage of food without affecting its taste.

pathogen Microorganism or virus that causes disease.

pathogenic bacteria See **pathogenic microorganism**.

pathogenic microorganism Organism capable of causing disease.

pathogenicity Ability of a microorganism to cause disease.

patient preoperative skin preparation Fast-acting, broad-spectrum, and persistent antiseptic-containing preparation that significantly reduces the number of microorganisms on intact skin.

pattern recognition receptor Protein capable of recognizing molecules frequently found on pathogens; can trigger host defense against pathogens.

pelvic inflammatory disease (PID) Acute or recurring acute infection of the oviducts and ovaries, with surrounding tissue involvement; includes inflammation of the cervix (cervicitis), uterus (endometritis), fallopian tubes (salpingitis), and ovaries (oophoritis), which can spread to adjacent connective tissue. The most common causes are *Chlamydia trachomatis* and *Neisseria gonorrhoeae*.

penicillin-binding protein (PBP) Transpeptidase enzyme important in bacterial cell wall formation. These proteins have various affinities to the β -lactam antibiotics and play an important role in resistance to these agents when altered.

penicillinase-producing *Neisseria gonorrhoeae* (PPNG) *N. gonorrhoeae* that have a type of penicillin resistance caused by genes located on the plasmids that code for penicillinase (a type of β -lactamase) production.

penicillinase-resistant penicillins Group of antimicrobial agents resistant to hydrolysis by β -lactamase, including oxacillin, methicillin, nafcillin, cloxacillin, and dicloxacillin.

peptidoglycan Unique mucopolysaccharide constituent of the bacterial cell wall. The quantity of this polymer and its location within the cell envelope is different between gram-negative and gram-positive bacteria.

performance improvement (PI) Continuous analysis of processes or procedures and continual implementation of improvements.

perinatal Refers to during labor and delivery of an infant; around the time of delivery.

peripheral chromatin DNA present on the nuclear membrane of some protozoa.

periplasmic flagella Fibrils around which the spirochete flexible cell wall is wound; also known as *axial fibrils*, *axial filaments*, *endoflagella*, and *periplasmic fibrils*; responsible for motility.

persistent Refers to prolonged or extended antimicrobial activity that prevents or inhibits the proliferation or survival of microorganisms after product application.

persister cell Microbial cell that is alive but weakly metabolic; it has the potential to return to an actively growing state.

persisters As related to minimal bactericidal concentration testing, the growth of small numbers (>0.1% of the test inoculum) of colonies from several concentrations of antimicrobial agent above the minimal inhibitory concentration.

personal protective equipment (PPE) Specialized clothing or equipment worn by an employee for protection.

pertussis Acute infectious inflammation of the airways characterized by recurrent bouts of spasmodic coughing, followed by a noisy inspiratory stridor, or "whoop." The infection is caused by *Bordetella pertussis* or *Bordetella parapertussis*.

pertussis toxin (PT) Protein exotoxin of *Bordetella pertussis* that produces a wide variety of responses in vivo. The main activity of pertussis toxin is modification of host proteins by ADP ribosyltransferase, which interferes with signal transduction.

petechiae Red spots or discoloration on the skin caused by minor bleeding underneath the skin. See also **purpura**.

phaeohyphomycosis Term used to define infections caused by fungi that produce dark cell walls from the presence of melanin.

phagocyte Mobile or fixed cell in a multicellular organism that is able to ingest debris. Phagocytes are present as "housekeepers" in all multicellular organisms. Neutrophils, macrophages, and histiocytes are the major human phagocytes.

phagocytosis Process of engulfing or ingesting and digesting foreign particles including bacteria.

pharyngitis Upper respiratory tract infection affecting the pharynx that may be caused by a virus or bacterium; acute bacterial pharyngitis is often caused by *Streptococcus pyogenes* and is characterized by sore throat, malaise, fever, and headache.

phenotype Observable or measurable characteristics of an organism.

pheromone Extracellular molecules passed between bacteria, allowing communication. When pheromones bind to specific receptors on bacteria, gene expression is altered.

- photochromogenic** Characteristic appearance of *Mycobacterium* spp. in which exposure to light stimulates pigment production.
- photochromogens** *Mycobacterium* spp. that produce carotene pigment on exposure to light.
- phylum (pl. phyla)** In biological classification, a subset or category.
- pigment** Coloring matter or substance.
- pilus (pl. pili)** Nonmotile, long, hollow protein tubes that connect two bacterial cells and mediate DNA exchange; also known as *conjugation pili*.
- pinta** Nonvenereal skin disease caused by *Treponema carateum*; found in the tropical regions of Central America and South America.
- planktonic** Free-floating microorganisms.
- plasmid** Extrachromosomal, circular pieces of DNA found in many strains of bacteria. Plasmids often carry virulence genes and antimicrobial resistance genes.
- plasmid profile analysis** Nonamplified typing method that analyzes the plasmid DNA profile from bacterial isolates.
- pleocytosis** Presence of white blood cells in cerebrospinal fluid.
- pleomorphic** Demonstrating a variety of shapes and forms; for a Gram stain, neither distinctly coccoid nor rod-shaped; also used to describe the Gram stain morphology of bacteria that exhibit a combination of cocci, bacilli, coccobacilli, and filamentous forms in a single stained smear.
- pleuropneumonia-like organism (PPLO)** After mycoplasmata were isolated from cows with pleuropneumonia, the first human mycoplasma was isolated and was referred to as a pleuropneumonia-like organism.
- pneumonia** Disease characterized by inflammation of the lungs, typically associated with fever, respiratory symptoms, and the presence of parenchymal involvement detected by physical examination or the presence of infiltrates on chest radiography.
- pneumonic plague** *Yersinia pestis* infection involving primary or secondary infection of the lungs.
- pneumonic tularemia** Pneumonia resulting from inhaling *Francisella tularensis*. This is the form that would most likely occur as a result of a bioterror release of this agent, although inhalation tularemia can occur naturally.
- polyclonal** Mixture of antibodies recognizing different antigens and epitopes with different binding affinities.
- polyclonal antibody** Mixture of antibodies able to bind the same antigen but that recognize different epitopes.
- polymerase chain reaction (PCR)** Molecular method that produces an exponential amount of product from a target nucleic acid sequence. PCR consists of denaturation, primer annealing, and primer extension, and it uses several components, including DNA polymerase, primers, target nucleic acid, a buffer, deoxynucleotides, and a thermal cycler.
- polymicrobial** Refers to the presence of two or more microbial morphotypes; if they are likely pathogens in an infection, the infection is polymicrobial.
- polymicrobial bacteremia** Bacteremia that involves more than one microbial organism.
- polymorphic fungus** Fungus that has both yeast and mold states present when cultured under the same growth conditions.
- polymyxin** Polymyxins B and E (also known as colistin) are antibiotics most effective against gram-negative bacteria. They work by damaging the bacterial cell membrane.
- polyvinyl alcohol (PVA)** Plastic resin often used in stool fixatives to adhere fecal material to a slide before staining.
- Pontiac fever** Mild febrile disease without pulmonary symptoms caused by *Legionella* spp.
- porin** Transmembrane structure made up of proteins large enough to facilitate passive diffusion.
- porphyrin** Intermediate substance formed during the biosynthesis of heme.
- positive predictive value (PPV)** See **predictive value**.
- postanalytic activity** Processes that occur after a sample has been analyzed, such as result review, transcription, or reporting of results.
- postsplenectomy** After removal of the spleen.
- postzone** Phenomenon resulting from an antibody solution that is too dilute to demonstrate agglutination or precipitation; also referred to as *antigen excess*.
- Pott disease** Name given to tuberculosis of the spine.
- preanalytic activity** Processes that occur before a sample is analyzed, such as patient identification and sample collection.
- precipitation** Immunologic reaction between soluble antigens and antibodies forming a visible macromolecular complex that falls out of solution.
- precision** Measure of exactness of a test.
- predictive value** Probability that a positive result (positive predictive value [PPV]) accurately indicates the presence of an analyte or a specific disease or that a negative result (negative predictive value [NPV]) accurately indicates the absence of an analyte or specific disease. Predictive values vary significantly with the prevalence of the disease or analyte unless the test is 100% sensitive (for NPV) or specific (for PPV).
- preseptal cellulitis** Inflammation of the periorbital tissues.
- prevalence** Number of cases of a disease that occur in a given moment in time or during a specific period in a given population.
- preventive maintenance** Procedures performed on instruments and equipment designed to keep them operating at optimal performance and to extend their lifetime.
- primary amebic meningoencephalitis** Infection of the central nervous system caused by the free-living amoeba, *Naegleria fowleri*.
- primary atypical pneumonia** Pneumonia that differs from the typical pneumonia caused by *Streptococcus pneumoniae*; primary refers to the fact that it is not preceded by another infection. Common causes of atypical pneumonia include *Legionella pneumophila*, *Chlamydia pneumoniae*, *Chlamydia psittaci*, and *Mycoplasma pneumoniae*. Atypical pneumonia caused by *Mycoplasma pneumoniae* is also known as walking pneumonia.
- primary bacteremia** Bacteremia that arises from an endovascular source, such as an infected cardiac valve or infected intravenous catheter.
- primary cell culture** Culture started from cells, tissues, or organs taken directly from an organism.
- primary syphilis** First stage of syphilis marked by the development of a chancre and spread of the causative spirochete in the tissues of the body.

primer Small, single-stranded oligonucleotide used in a polymerase chain reaction or other molecular diagnostic method. Primers anneal to target sequences, and DNA polymerase starts the synthesis of new DNA strands from the primers.

primer annealing Second step of a polymerase chain reaction, in which primers anneal to denatured target nucleic acid strands. This hybridization reaction usually occurs at a temperature of 5° C lower than the melting temperature (T_m) of the primers.

primer-dimers Primers that anneal to each other and form hybrids during a polymerase chain reaction or other amplification procedures.

primer extension Third step of a polymerase chain reaction. Once primers have annealed to template nucleic acid, DNA polymerase uses free deoxynucleotides and synthesizes new strands of DNA from the primers.

prion Infectious proteinaceous particle. These agents are known to cause degenerative diseases of the nervous system (transmissible spongiform encephalopathies), such as Creutzfeldt-Jakob disease in humans.

probe Oligonucleotide with an attached label for detection, often a fluorescent chemical. Probes anneal to target sequences in many molecular diagnostic reactions.

probe-mediated stain Relatively simple chemical stain used to make antibody- or nucleotide-mediated identity reactions visible.

process monitor Ongoing data collection that can be used to establish trends or spot problems when trends are disrupted.

proctitis Rectal inflammation that can occur as a primary manifestation of infection following direct inoculation of the rectal mucosa.

proficiency testing Testing of carefully designed, “unknown” samples to evaluate analytic processes.

proglottid One segment of a tapeworm. Each proglottid contains both male and female reproductive organs.

prokaryote Microorganisms that lack a true nucleus and nuclear membrane and organelles.

prostatitis Inflammation in the prostate.

protective antigen One of three proteins that make up the anthrax toxin. Protective antigen is necessary for edema factor and lethal factor to bind to and penetrate host cells.

protein A Cellular component of *Staphylococcus aureus* that can bind immunoglobulin G and prevent phagocytosis.

protein expression Process whereby a gene's DNA sequence is converted into a structure or function of a cell; also called *gene expression*.

proteomics Large-scale analysis of protein expression, often at the cellular level. This method is often used to analyze proteins expressed in various disease states.

prozone Phenomenon resulting from a high concentration of antibodies preventing agglutination or precipitation; also referred to as *antibody excess*.

pseudobacteremia Seen when bacteria are recovered from blood cultures; might be caused by contamination of blood samples during phlebotomy, leading to false-positive results.

pseudo-germ tube Tubelike projection from a blastoconidium or spore, with a constriction at its base.

pseudohypha (pl. pseudohyphae) Chain of elongated fungal cells with constrictions at points of attachment resembling true hyphae.

pseudomembranous colitis See *Clostridioides difficile*-associated disease.

pseudomonad Gram-negative, nonfermentative, oxidase-positive bacillus, possessing polar flagella; found in most aquatic environments; not usually part of the normal human biota.

psychrophile Organism that grows best at cold temperatures (optimal growth at 10° to 20° C).

public health The scientific discipline that pertains to protecting the safety and health of people and their communities.

pulsed-field gel electrophoresis (PFGE) Commonly used strain-typing method. A restriction enzyme is used to digest chromosomal DNA, and the resulting fragments are separated in an agarose gel in a pulsed electric field. The strains show unique banding patterns.

purified protein derivative (PPD) Diagnostic skin test for tuberculosis containing proteins from *Mycobacterium tuberculosis*.

purpura Purplish discoloration that appears under the skin, caused by bleeding underneath the skin. Small spots are called *petechiae*; large areas are referred to as *ecchymoses*.

purpura fulminans Skin manifestation of disseminated intravascular coagulation (DIC) that is classically associated with meningococcemia, but has also been associated with *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Haemophilus influenzae* bloodstream infections. It is characterized by rapidly developing skin hemorrhage and necrosis and peripheral gangrene accompanied by shock syndrome.

purulence Seen grossly as pus, consisting of neutrophils, protein, and necrotic debris.

pyelonephritis Inflammation that involves the kidneys.

pyocyanin Water-soluble blue pigment, characteristic of *Pseudomonas aeruginosa*. When combined with the fluorescein pigment of *P. aeruginosa*, it forms blue-green colonies on a variety of solid media.

pyoderma Skin infection characterized by the appearance of pus and necrosis in tissues.

pyogenic streptococci See *Streptococcus pyogenes*.

pyoverdin Fluorescein pigment of members of the *Pseudomonas* fluorescent group. Fluorescein can be seen with ultraviolet light. When combined with the pyocyanin (blue) pigment of *P. aeruginosa*, a blue-green colony results.

pyrosequencing Sequencing by synthesis technique that does not require labeled nucleotides or primers and also does not require a postreaction electrophoresis step. It is a rapid sequencing technique that generates approximately 20 to 50 base sequences per primer, so this technique is best used for short sequences. Pyrosequencing uses the enzymes DNA polymerase, ATP sulfurylase, luciferase, and apyrase and the substrates adenosine 5'-phosphosulfate (APS) and luciferin.

pyrrolidonyl- α -naphthylamide (PYR) hydrolysis Test used for the presumptive identification of group A streptococci (positive) and enterococci (positive). The substrate is hydrolyzed to β -naphthylamine by the enzyme pyrrolidonyl arylamidase.

pyuria Presence of white blood cells (leukocytes) in the urine; more than 8 to 10 leukocytes/mm³ indicates significant pyuria.

Q

Q fever See **query fever**.

Q probes External peer comparison program sponsored by the College of American Pathologists that addresses process, outcome, and structure-oriented quality assurance issues.

QRNG Fluoroquinolone-resistant *Neisseria gonorrhoeae*.

quality assurance (QA) A process that involves monitoring all components of a system or procedure (preanalytical, analytical, and postanalytical) and implementing changes when suboptimal performance is identified, involves taking proactive measures to ensure high-quality patient care.

quality control (QC) System for detecting and correcting analytic errors by establishing performance limits and ensuring the maintenance of proper standards, especially by periodic random inspection of the product.

quantitative isolation Technique in which a measured amount of specimen is inoculated to an agar plate to determine the quantity of microbes in the specimen.

query fever Original name of Q fever, caused by *Coxiella burnetii*.

quinolone Class of broad-spectrum antimicrobial that acts by inhibiting the bacterial enzyme DNA gyrase or topoisomerase IV enzyme, thereby inhibiting DNA replication and killing the organism.

quorum sensing Process whereby bacteria communicate by means of extracellular molecules called *pheromones*.

quorum-sensing inhibitor (QSI) Molecule that can interfere with the quorum sensing system of bacteria. They have been used in an attempt to disrupt biofilms.

R

radial immunodiffusion (RID) Precipitation method with antibody evenly distributed in agarose and antigen added to wells. As antigen diffuses, it forms a concentration gradient. At some point it forms a zone of equivalence with the antibody and a precipitate forms. The precipitant ring is proportional to antigen concentration.

radioimmunoassay (RIA) Immunoassay that uses radioactive isotopes to detect antigen-antibody complexes in vitro; rarely used today in clinical laboratories.

random amplified polymorphic DNA (RAPD) Amplified strain-typing procedure that uses random sequence primers that anneal to random chromosomal DNA sequences of the strain of interest; a PCR assay is used to amplify DNA, and strains have unique patterns of fragments.

rapid diagnostic test (RDT) An easy-to-use test that provides quick results, usually in 20 minutes or less; many are point-of-care tests.

rapid plasma reagin (RPR) test Nontreponemal serologic test for syphilis.

RASE Term used to describe prescription drug products that are "recognized as safe and effective."

reagin antibody (RB) Antibody produced during *Treponema pallidum* infection against nontreponemal antigens. Immunoglobulin E antibodies cause a type 1 hypersensitivity reaction; also referred to as *reagin*.

real-time polymerase chain reaction (PCR) Commonly used detection method for PCR amplicons. A probe or fluorescent dye is used for detection. The real-time PCR platform analyzes the accumulation of fluorescence after every PCR cycle so the results can be observed on a computer screen in real time. Thus PCR and detection are coupled from the same tube.

reemerging pathogens Microbial pathogens, once thought to be eliminated or greatly reduced in numbers, that are seen once again or potentially seen.

Regan-Lowe (RL) transport medium Recommended transport medium for suspected *Bordetella pertussis* when overnight or several-day transport is required; contains charcoal (half-strength from charcoal horse blood agar isolation media), 10% horse blood, and 40 mg/L cephalaxin.

Reiter syndrome Disorder that causes urethritis, conjunctivitis, polyarthritides, and mucocutaneous lesions; in adults it is believed to be caused by *Chlamydia trachomatis*.

relapsing fever Can be tickborne (endemic relapsing fever) or louseborne (epidemic relapsing fever); caused by *Borrelia* spp. Relapsing fever is best prevented by control of exposure to the arthropod vectors.

relebactam A β -lactamase inhibitor approved for use in combination with imipenem/cilastatin.

repetitive palindromic extragenic element polymerase chain reaction (Rep-PCR) Form of strain typing using primers complementary for repetitive palindromic sequences that occur throughout chromosomes. PCR is used to amplify DNA between the palindromic sequences. Strains have unique fragment patterns.

resident microbial biota Microorganisms usually found at a particular body site of healthy individuals and that remain at the site for long periods or indefinitely.

resistant strain In therapeutic terms, these strains are not inhibited by the usual systemic concentrations of the antimicrobial agent with normal dosage schedules or fall in the range in which specific microbial resistance mechanisms are likely and clinical efficacy has not been reliable in treatment studies.

respiration The efficient, energy-generating process in which molecular oxygen is the final electron acceptor; also called *oxidation*.

respiratory burst Release of reactive oxygen species (superoxide radical and hydrogen peroxide) from different types of cells. It usually denotes the release of these chemicals from immune cells (e.g., neutrophils, macrophages) as they come into contact with different bacteria or fungi.

restriction enzyme Enzyme that cuts DNA at specific deoxyribonucleotide sequences. Many unique restriction enzymes are commercially available. They are used in various molecular techniques, such as Southern blotting; also called *restriction endonuclease*.

reticulate body (RB) Metabolically active noninfectious form of *Chlamydia*.

retinitis Inflammation of the retina.

reverse passive agglutination test Agglutination immunoassay in which antibodies are attached via the Fc portion of the antibody to a carrier particle such as latex.

reverse transcription–polymerase chain reaction (RT-PCR) Type of PCR that uses reverse transcriptase to produce cDNA copies of transcript. The cDNA is then used in a standard PCR assay using specific primers. RT-PCR is used to assay for RNA viruses and analyze transcript.

rhabditiform larva Feeding but noninfective larval stage; this stage hatches from the egg.

rheumatic fever A sequela of *Streptococcus pyogenes* pharyngitis characterized by fever and inflammation of the heart, joints, blood vessels, and subcutaneous tissues.

rheumatoid factor (RF) Autoantibody, usually immunoglobulin M, directed against abnormal immunoglobulin G antibodies.

rhinocerebral mucormycosis Infection of the sinuses, nasal passages, oral cavity, and brain caused by saprophytic fungi. It usually affects individuals with diabetes and those who are immunocompromised. The infection can rapidly result in death.

rhizoid Rootlike hypha.

ribonuclease H (RNase H) Ribonuclease that degrades RNA in DNA-RNA hybrids.

ribotyping Method that detects rRNA restriction fragment length polymorphism patterns by Southern blotting. It displays excellent reproducibility and discriminatory power because the genes that code for rRNA are conserved in different species of organisms and appear in conserved positions of a species chromosome.

ricin Derived from the seeds of castor oil plant, a potent biological toxin that inhibits protein synthesis.

risk group Categorization for infectious agents based on hazardous characteristics and relative risk.

Ritter disease Disorder also known as *staphylococcal scalded skin syndrome*.

river blindness Disease caused by *Onchocerca volvulus*; also called *onchocerciasis*.

Rocky Mountain spotted fever (RMSF) Most severe of the rickettsial infections; the most common in the United States. Humans acquire the infection by tick bites; caused by *Rickettsia rickettsii*.

rolling circle amplification (RCA) An isothermal enzymatic amplification strategy to synthesize ultralong single-stranded DNA.

rotavirus Member of the family Reoviridae that infects cells of the villi of the small intestine, leading to epithelial atrophy and proliferation of cells with secretory capacity. This may decrease the absorptive capacity of the bowel, contributing to diarrhea. Rotaviruses are the major cause of diarrhea in children younger than 5 years of age.

S

safety data sheet (SDS) See **material safety data sheet**.

salpingitis Inflammation of the fallopian tubes.

Sanger sequencing DNA sequencing method utilizing the random incorporation of chain-terminating dideoxynucleotides by DNA polymerase during in vitro DNA replication followed by electrophoresis.

saprobe Environmental fungus that derives nutrients from dead organic material; generally nonpathogenic for humans.

satellitism Small colonies of bacteria growing around another colony; the small colonies derive an essential nutrient secreted by the other colony; phenomenon exhibited by some *Haemophilus* spp. on sheep blood agar.

scalded skin syndrome (SSS) Toxin-mediated exfoliative dermatitis associated with *Staphylococcus aureus*, superficially resembling a burn injury.

scarlet fever Disease characterized by a diffuse red rash that appears on the upper chest and spreads to the trunk and extremities; caused by strains of *Streptococcus pyogenes* that produce an erythrogenic toxin.

schizogony Asexual life cycle of the Apicomplexa. In malaria, it is characterized by splitting of the nucleus into multiple segments and the formation of merozoites.

schizont Parasitic form producing merozoites during schizogony. In malaria, it is part of the erythrocytic asexual life cycle.

Schüffner stippling Malarial pigment characterized as tiny red staining dots in red blood cells infected with *Plasmodium vivax* or *P. ovale*.

scleritis Inflammation of the sclera, the white outer layer of the eyeball.

scolex Anterior end of a tapeworm that contains structures such as suckers or hooklets used for attachment to the host.

scombroid Form of food poisoning from ingestion of heat-stable toxins produced by bacteria in contaminated fish—often tuna, mackerel, or yellow jack.

Scorpion primer Used with a real-time polymerase chain reaction (PCR) assay. A Scorpion primer is a hairpin loop structure with a fluorophore on the 5' end and a quencher on the 3' end. Also attached to the 3' end is a small primer sequence specific for the target of interest. The primer anneals to target nucleic acid, and primer extension occurs. Denaturation dissociates the PCR product and the Scorpion primer. A portion of the primer is complementary to the PCR product and anneals to it, physically separating the fluorophore from the quencher, and an increase in fluorescence is observed.

scotochromogenic Refers to *Mycobacterium* spp. producing pigment in a light or dark growth environment.

secondary bacteremia Bacteremia that arises from an infected extravascular source such as the lung in patients with pneumonia.

secondary syphilis Second stage of syphilis that appears 2 to 6 months after primary infection; marked by lesions, especially in the skin, but also in organs and tissues; lasts from 3 to 12 weeks.

select agent Biological agent or toxin deemed by the U.S. Department of Health and Human Services to have the potential to pose a serious threat to public health and safety and to animal health and animal products.

select biological agent Microbial agents classified by the Centers for Disease Control and Prevention as potential agents of bioterrorism because of their dissemination potential and ability to cause disease and/or death in humans, animals, and plants.

selective media Media that support the growth of one type or one group of microbes but not of another type.

selective reporting Reporting of results for certain antimicrobial agents based on defined criteria such as organism identification, body site, and overall susceptibility profile following testing of a clinical isolate. Secondary (broader-spectrum, more costly, more toxic) agents are only reported if primary agents are resistant or if they offer significant clinical advantages for the particular isolate and patient.

sentinel laboratories Part of the Laboratory Response Network; composed of most of the hospital-based microbiology laboratories.

sepsis Systemic response to bacterial infection.

septic shock Sepsis accompanied by refractory hypotension.

septicemia Bloodstream infection. See **sepsis**.

sequencing Determining the primary structure of an unbranched biopolymer in genetics and biochemistry. Sequencing results in a symbolic linear depiction, known as a *sequence*, that succinctly summarizes much of the atomic level structure of the sequenced molecule. DNA sequencing is the process of determining the nucleotide order of a given DNA fragment.

seroconversion Demonstration of detectable antibodies to an immunogen, such as an infectious agent or vaccine.

serology Study of serum or, more specifically, the use of immunologic assays to detect antibodies for the purpose of diagnosing an infectious disease.

serotype Antigenic constituency of an organism.

serotyping Example of phenotypic testing used with biochemical testing for identifying or classifying different strains of bacteria within a species. Serotyping uses antibodies to detect specific antigens on the surface of bacteria; also called *serogrouping*.

serum bactericidal test (SBT) Method for determining the highest dilution or titer of a patient's serum that is inhibitory to the patient's own infecting bacterium and the highest dilution or titer bactericidal to the patient's own infecting bacterial isolate.

sessile Microorganisms attached to a solid surface, usually found in a community of cells called a *biofilm*.

sexual reproduction Union of a male and a female cell to produce a zygote.

Shiga toxin (Stx) One of two toxins produced by *Shigella* and enterohemorrhagic *Escherichia coli* strains, toxic to African green monkey kidney (vero) cells.

Shiga-toxin producing *Escherichia coli* Strain of *E. coli* that produces Shiga toxin 1 and/or Shiga toxin 2, resulting in gastroenteritis.

shock Serious, life-threatening medical condition in which insufficient blood flow reaches the body tissues.

simple stains Chemical stains that are water- or alcohol-based that can be used directly on a fixed or air-dried sample

without additional preparation or complex steps. Gram, Wright, and acridine orange are simple stains.

sinusitis Disease that results from infection of one or more of the paranasal sinuses.

Six Sigma (6σ) A tool for process improvement that designates how often mistakes are likely to occur in a process or procedure and represents the number of errors per million opportunities—the higher the Sigma, the fewer the number of errors. A Sigma score of six is the highest level that can be achieved.

skipped wells As related to broth dilution minimal inhibitory concentration testing, refers to growth at higher concentrations and no growth at one or more of the lower concentrations of antimicrobial agent in the series of concentrations tested.

small colony variants Rare, fastidious strains of staphylococci requiring CO₂, hemin, or other complex nutrient for growth; grow on media containing blood, forming colonies about 10% of the size of wild-type strains after incubation for at least 48 hours.

smooth Characterization of some edges of colonies; having an even and regular surface or consistency.

sodium polyanethol sulfonate (SPS) Anticoagulant used in collection of microbiology specimens. In addition to preventing clot formation, it is anticomplementary and antiphagocytic.

Southern blot Hybridization procedure in which chromosomal DNA is digested with a restriction enzyme and the fragments are separated by agarose gel electrophoresis. A specific, labeled probe is then used to detect target DNA.

species In microbiology (biology), it is the basic unit of biodiversity. In classifying organisms, it is assigned a two-part name in Latin; the genus is listed first, followed by a specific species name.

spirochetes Slender, flexuous, helically shaped, unicellular bacteria ranging from 0.1 to 0.5 μm wide and from 5 to 20 μm long, with one or more complete turns in the helix.

sporangiophore Hypha stalk upon which a sporangium develops; the stalk may be branched or unbranched.

sporangiospore Asexual spore produced in a sporangium.

sporangium (pl. sporangia) Closed sac-like structure containing asexual spores.

spore In fungi, a unit of asexual reproduction that may be adapted for dispersal and survival, often for extended periods, in unfavorable conditions. In bacteria, spores are a means of surviving harsh conditions; they are not a means of reproduction. In bacteria, spores appear as highly refractile bodies in the cell. They are visualized microscopically as unstained areas in the cell when using traditional bacterial stains (Gram). May be stained with specific spore stains.

sporocidal Refers to a substance or product that kills spores.

sporoblast Structure within an immature oocyst that ultimately develops into a sporocyst.

sporocyst Structure within the oocyst that contains the sporozoites.

sporogony Sexual life cycle of malaria that takes place in the gut of the mosquito; results in the production of sporozoites, the infective stage for humans.

sporozoite Elongated cell that develops within an oocyst. In malaria, it is the stage transmitted to humans when the mosquito takes a blood meal. In other members of the Apicomplexa, it is contained within the oocyst that is passed in the feces.

standard precautions Infection control precautions that assume that all patients are potentially infectious; practices are implemented to prevent contact with blood and body fluids.

staphylocoagulase See coagulase.

staphylococcal enterotoxins Small-molecular-weight polypeptides belonging to the bacterial superantigen family.

staphylococcal scalded skin syndrome See scalded skin syndrome.

static Term indicating that a substance inhibits the growth of an organism but that on removal the organism might grow again.

sterile pyuria Presence of 10 white blood cells per high-power field seen in centrifuged urine samples without any uropathogens recovered from urine cultures.

sterilization Refers to the destruction of all forms of life, including bacterial spores.

strain Variant of a plant, bacterium, or virus.

strand displacement amplification (SDA) An isothermal assay that can detect trace amounts of DNA or RNA.

streamers Growth of an organism in liquid media, represented by vines and puffballs.

streptococcal pyrogenic exotoxin Toxin produced by some strains of *Streptococcus pyogenes*; can result in scarlet fever.

streptogramin Antibiotic mixture of two structurally distinct compounds, type A and type B, that are separately bacteriostatic but bactericidal in appropriate ratios. These antibiotics act at the level of translation inhibition through binding to the bacterial ribosome. Quinupristin and dalfopristin are both streptogramins.

streptolysin O (SLO) Oxygen-labile hemolysin produced by *Streptococcus pyogenes* responsible for hemolysis on sheep blood agar plates incubated anaerobically.

streptolysin S Streptococcal hemolytic exotoxin that is oxygen-stable, lyses leukocytes, and is nonimmunogenic. The hemolysis seen around colonies that have been incubated aerobically is caused by streptolysin S.

string of pearls Description of the microscopic appearance of *Bacillus anthracis* after short-term exposure to penicillin. The presence of large spherical organisms in chains is useful for a presumptive identification.

stringency Strictness of binding of complementary single-stranded nucleic acid molecules modulated by altering the hybridization conditions.

stye An infection involving one or more of the small glands near the base of an eyelash, usually caused by bacteria.

suboptimal specimen Specimen that is not properly selected, collected, or transported and will lead to misleading results in a microbiology workup.

sulbactam A β -lactamase inhibitor used with ceftriaxone.

sulfamethoxazole (SMZ) Antimicrobial that blocks the step leading to the formation of 7,8-dihydropteroate by competitively inhibiting the binding of the structural

analogue *p*-aminobenzoic acid with dihydropteroate synthase.

sulfide-indole-motility medium (SIM) Bacteriologic medium used to determine whether an organism is motile and has the ability to produce H_2S and form indole from tryptophan.

sulfur granules Masses of filamentous organisms mixed with inflammatory debris that may appear yellow or orange seen in certain mycetomas.

superoxide anion Oxygen free radical, O_2^- , toxic to cells.

superoxide dismutase Enzyme composed of metal-containing proteins; converts superoxide radicals into less toxic agents.

suppurative Forming or discharging pus.

surgical hand scrub Antiseptic-containing preparation that significantly reduces the number of microorganisms on intact skin; it is broad-spectrum, fast-acting, and persistent.

surgical site infection (SSI) Infection that occurs at a surgical site (incision).

surveillance Ongoing systematic collection of data; analysis and interpretation of details surrounding a disease or event.

surveillance culture Testing of selected patients or environmental sources to identify the source of infectious agents.

susceptible In therapeutic terms, implies that a bacterial strain tested is inhibited or killed by an antimicrobial agent and the infection it is causing may be appropriately treated with the dosage of antimicrobial agent recommended for that type of infection and infecting species.

swarming Refers to a hazy blanket of growth on the surface that extends well beyond the streak lines.

swimmer's itch Dermatitis caused by the larvae of certain schistosomes of birds and mammals that may penetrate the human skin.

SYBR Green Dye that binds to nucleic acids; fluoresces green after exposure to ultraviolet light. SYBR Green I is used to stain DNA, and SYBR Green II is used to stain RNA.

Sydenham chorea A neurological disorder linked to *Streptococcus pyogenes* infection.

symbionts Organisms that share a relationship; typically at least one member of the pair benefits from the relationship.

symbiosis Relationship (interaction) between species (host and organism) in which typically at least one benefits from the relationship.

synanamorph Two or more different fungi that reproduce asexually (anamorphs) but have been linked to the same sexual fungus (telomorph).

syncytia Giant multinucleated cells formed from cell fusion as a result of virus infection.

synergism Occurs when the antimicrobial activity of a combination of antimicrobial agents is greater than the activity of the individual agents alone.

syphilis Multistage sexually transmitted disease caused by *Treponema pallidum* subsp. *pallidum*.

syphilitic chancre Initial lesion of syphilis that is painless and nonsuppurative but infectious; its appearance signals primary syphilis. The chancre appears at the site of inoculation, usually the genitalia.

systemic inflammatory response syndrome

(SIRS) Inflammatory state affecting the whole body related to sepsis; SIRS comprises a spectrum of increasingly severe conditions ranging from sepsis to severe sepsis to septic shock.

T

T-helper cells Cells that express CD4 and a T-cell receptor on their surfaces and respond to antigen presentation by antigen-presenting cells.

T-strain mycoplasma Original name given to the *Ureaplasma* spp. because they produce tiny colonies compared with the *Mycoplasma* spp.

tache noire Literally “black spot”; characteristic lesion that can form when *Rickettsia conorii* or *Orientia tsutsugamushi* organisms infect humans; formed at the arthropod vector bite site.

tachyzoite Motile, replicating intracellular stage of *Toxoplasma gondii*.

target Nucleic acid species studied in molecular diagnostics assays. The target is single-stranded and complementary to primers and/or probes.

targeted surveillance Surveillance of specific, preidentified events of concern or importance.

taxa Term that denotes categories of organisms.

taxonomy Orderly classification and grouping of organisms into taxa or categories.

tazobactam A β -lactamase inhibitor used with piperacillin.

teleomorph Form of a fungus that reproduces sexually.

temperate Phage DNA that becomes incorporated into the bacterial genome, where it is replicated along with the bacterial chromosomal DNA.

template Target nucleic acid for molecular diagnostics assays.

tertiary syphilis Third stage of syphilis; develops after the disappearance of the secondary symptoms, marked by ulcers in and gummas under the skin and commonly by involvement of the skeletal, cardiovascular, and nervous systems.

test validation Ongoing process providing information that a test is performing correctly.

tetanospasmin Neurotoxin that interferes with the release of neurotransmitters, blocking inhibitor impulses, leading to unopposed muscle contraction and spasm, the characteristic signs and symptoms of tetanus produced by *Clostridium tetani*.

tetanus Disease that acts on the central nervous system; characterized by muscular contractions See **tetanospasmin**.

tetracycline Broad-spectrum antibiotic produced by the *Streptomyces* spp., indicated for treatment of various bacterial infections. It inhibits cell growth through inhibition of translation by binding to the 30S ribosomal subunit and preventing the aminoacyl tRNA from binding to the A site of the ribosome.

The Joint Commission (TJC) Health care accrediting organization whose mission is to improve the safety and quality of care provided to the public.

thermal cycler Programmable instrument that cycles temperatures for PCR assays.

thermophiles Bacteria that grow best at high temperatures (optimal growth at 50° to 60° C).

thiosulfate citrate bile salts sucrose (TCBS) agar Selective medium used for isolation of *Vibrio* spp. Sucrose-fermenting species are yellow; nonsucrose-fermenting organisms are green.

thrush Yeast infection occurring in the oral mucosa, generally found in patients with a cell-mediated immune deficiency.

tigecycline The first drug in the glycylcycline class of antibiotics, related to tetracycline.

Tier 1 agent Designation applied to biological agents and toxins that present the greatest risk of deliberate misuse with significant potential for mass casualties or devastating effect to the economy, critical infrastructure, or public confidence, and pose a severe threat to public health and safety.

time-kill assay Method for measuring the rate of killing of a bacterial isolate by an antimicrobial agent by examining the number of viable bacteria remaining at various intervals after exposure to the agent.

tinea Term used to denote superficial fungal diseases of various parts of the body; also called *ringworm*.

tissue culture Maintenance or growth of complex tissue.

T_m See **melting temperature**.

tolerance As related to minimal bactericidal concentration (MBC) testing, the lack of bactericidal activity at several concentrations above the minimal inhibitory concentration (MIC), often defined as an MBC/MIC ratio of 32:1 or higher.

total quality management (TQM) A management framework based on the belief that an organization can build long-term success by having all its members, from low-level workers to its highest ranking administrators, focus on improving quality and thus delivering customer satisfaction.

total surveillance Surveillance of all events in a health care setting.

toxic epidermal necrolysis (TEN) Clinical manifestation of a disorder with multiple causes, often a drug or chemical reaction; may resemble scalded skin syndrome.

toxic megacolon Life-threatening complication of intestinal conditions characterized by a very inflated colon, abdominal distention, fever, pain, and shock.

toxic shock syndrome (TSS) Potentially fatal multisystem disease caused by toxins, primarily TSST-1, produced by *Staphylococcus aureus*.

toxic shock syndrome toxin-1 (TSST-1) Exotoxin acting as a superantigen; usually associated with toxic shock syndrome.

tracheal cytotoxin Virulence factor of certain *Bordetella* spp. that contributes to pathogenesis by causing ciliostasis, inhibiting DNA synthesis, and promoting cell death.

trachoma Chronic severe eye infection associated with specific serovars (A, B, Ba, and C) of *Chlamydia trachomatis*.

trailing As related to broth dilution minimal inhibitory concentration testing, growth at lower concentrations followed by one or more wells or tubes that show greatly reduced growth in the form of a small button or light haze.

transcript Messenger RNA, the expressed product of a gene.

transcription Process whereby a template DNA strand is copied into a functional RNA sequence, resulting in mature mRNA or structural RNA.

transcription-mediated amplification (TMA) Amplification assay similar to nucleic acid sequence–based amplification.

transduction Transfer of bacterial genes by a bacteriophage (bacterial virus) from one cell to another.

transformation Uptake and incorporation of naked DNA into a bacterial cell.

transient bacteremia Bacteria in the bloodstream sometimes occurring after procedural manipulation of a particular body site colonized by indigenous biota.

transient microbiota Microorganisms that reside at body sites for short periods, sometimes for days or weeks. Not usually part of the established microbiota.

transillumination Passing of bright light through the bottom of the plate to determine whether the organism is hemolytic. This technique can be also used on clear media (e.g., MacConkey), to see slight differences in the color of nonlactose-fermenting bacilli.

translation As in genetics, the process of using a DNA sequence (gene) to make proteins.

translucent Permitting light to pass through but diffusing it so objects on the opposite side are not clearly visible.

transmission-based precautions Extra steps are needed to prevent the spread of an infectious agent when a patient is known or suspected to be infected or colonized with an infectious agent.

transparent Having the property of transmitting light rays through a substance so objects situated beyond or behind can be seen distinctly.

transport medium Liquid or semi-solid medium meant to preserve and maintain the integrity of infectious agents in a clinical specimen for the period between specimen collection and laboratory processing.

transposon (Tn) Segment of DNA that can become integrated at many different sites along a chromosome, especially a segment of bacterial DNA that can be translocated as a whole.

traveler's diarrhea Acute diarrheal illness generally acquired when travelers ingest contaminated food or water; often caused by enterotoxigenic *Escherichia coli*; also known as *turista*.

treponemal antibody tests Tests for syphilis that detect specific antibodies to treponemal antigens used to confirm a nontreponemal antibody screening test.

trichomoniasis Extremely common sexually transmitted infection; common manifestation of vaginitis. *Trichomonas vaginalis* is the causative agent of trichomoniasis.

trimethoprim (TMP) An antimicrobial agent that interferes with folic acid metabolism; It is often used with sulfamethoxazole.

triple sugar iron (TSI) agar Bacteriologic medium that aids in the determination of carbohydrate fermentation (glucose, lactose, and sucrose) and H₂S formation.

trophozoite Feeding, motile, noninfective form of a protozoan. This form replicates in the host and is responsible for causing damage.

true pathogen Microorganism capable of producing disease in immunocompetent and immunocompromised individuals; sometimes referred to as *overt pathogen*.

trypomastigote Life-cycle stage found in humans characteristic of blood and tissue flagellates; long, spindle-shaped organism with a flagellum, undulating membrane, prominent nucleus, and kinetoplast.

tuberculous meningitis Meningitis or inflammation of the meninges from an infection caused by *Mycobacterium tuberculosis*.

tularemia Disease caused by the bacterium *Francisella tularensis*.

tumbling motility Characteristic end-over-end motility of *Listeria* spp.

turbidity Cloudiness of liquid media caused by growth of microorganisms.

turista See **traveler's diarrhea**.

type B gastritis Type of chronic gastritis usually associated with *Helicobacter pylori* infection.

U

ulceroglandular tularemia Most common form of tularemia; characterized by a skin ulcer at the site of entry and swollen region lymph nodes..

umbilicate Depressed center, concave.

umbonate Raised or bulging center, convex.

undulant fever Synonym for *brucellosis*; rising and falling fevers.

undulating membrane A cytoplasmic membrane associated with some flagellates that extends out like a fin of the outer edge of the cell, moving in a wavelike fashion.

uracil-N-glycosylase (UNG) Enzyme that prevents replication of DNA strands synthesized with uracil instead of thymine. UNG is sometimes used as a contamination prevention measure.

urea breath test Rapid diagnostic procedure used to identify infections by *Helicobacter pylori*, a bacterium implicated in gastritis, gastric ulcers, and peptic ulcer disease; patients are given an oral dose of radioactive urea. In patients with *H. pylori* infection, radioactive carbon dioxide can be detected in the breath.

urease test Bacteriologic test to determine an organism's ability to break down urea with the enzyme urease.

ureteritis Inflammation of one or both ureters, ducts that carry urine from the kidneys to the bladder.

urethritis Inflammation of the urethra.

urinary tract infection (UTI) Infection occurring in or associated with the urinary tract.

usual (indigenous) microbiota Microorganisms usually found at body sites without causing disease; certain species may produce disease, given the opportunity, or when the host's immune system is weakened.

uveitis Inflammation of the iris, ciliary body, and choroidal tissues.

V

V factor (nicotinamide adenine dinucleotide [NAD]) Growth factor that some *Haemophilus* spp. require in media for growth; also known as *nicotinamide adenosine dinucleotide*.

vaccinia virus Virus strain used to vaccinate humans against smallpox.

vasculitis Widespread inflammation of small blood vessels.

vancomycin agar screen plate Brain-heart infusion agar plate containing 6 µg/mL of vancomycin; used for detection of vancomycin-resistant enterococci, vancomycin-intermediate *Staphylococcus aureus*, and vancomycin-resistant *S. aureus*.

vancomycin-intermediate *Staphylococcus aureus* (VISA) *S. aureus* isolates that show reduced susceptibility levels to vancomycin and fall in the intermediate range of 8 to 16 µg/mL.

vancomycin-resistant enterococci (VRE) Enterococci that have vancomycin minimal inhibitory concentration in the resistant range ≥ 32 µg/mL.

vancomycin-resistant *Staphylococcus aureus* (VRSA) *S. aureus* organisms that have vancomycin minimal inhibitory concentration in the resistant range ≥ 32 µg/mL.

variola major Severe form of smallpox, with approximately 30% mortality.

variola minor Milder form of smallpox, with approximately 1% mortality.

vector Organism responsible for transmitting parasite from infected host to noninfected host.

Venereal Disease Research Laboratory (VDRL)

test Nontreponemal antigen test commonly used for the diagnosis of syphilis, named after the Venereal Disease Research Laboratory where the test was developed.

ventilator-associated pneumonia (VAP) Pneumonia whose cause has been associated with a ventilator device, such as an endotracheal tube or a tracheotomy.

verotoxin See Shiga toxin.

vesicles Liquid-filled sacs.

Vi antigen See K antigen.

viremia Presence of viruses in the blood.

virion Complete virus particle.

virucidal Antimicrobial that inactivates viruses.

virulence Degree of pathology caused by an organism. The extent of the virulence is usually correlated with the ability of the pathogen to multiply within the host; may be affected by other factors; the disease-evoking power of a microorganism.

Voges-Proskauer (VP) test See methyl red–Voges-Proskauer (MRVP) test.

vulvovaginal candidiasis Symptomatic vaginitis usually caused by infection with the yeast *Candida*.

W

Waterhouse-Friderichsen syndrome Complication of *Neisseria meningitidis* infection involving hemorrhage of the adrenal glands; characterized by a sudden onset of fever, cyanosis, petechial hemorrhages of the skin and mucous membranes, and coma.

Weil disease Severe systemic form of leptospirosis that includes renal failure, hepatic failure, and intravascular disease; may result in death.

Weil syndrome Synonym for icteric leptospirosis.

Whipple disease Rare intestinal disease caused by *Tropheryma whippelii*; characterized by severe intestinal malabsorption.

whooping cough See pertussis.

wool sorter's disease Name given to inhalation (pulmonary) anthrax describing anthrax disease associated with occupational exposure to *Bacillus anthracis* spores as a result of handling contaminated animal products.

work practice controls Alterations in the manner in which a task is performed to reduce the likelihood of exposure to infectious agents.

X

X factor Growth factor that some *Haemophilus* spp. require in media for growth; also known as *hemin* or *hematin*.

Y

yaws Nonvenereal spirochetal disease of the tropics resembling syphilis; caused by *Treponema pallidum* subsp. *pertenue*.

yeast Fungus that reproduces by budding.

Z

zeta potential Electrical or charge potential on cells. As in immunoassays, erythrocytes have a negative charge and will repel each other. In order to exhibit hemagglutination, antibody-antigen interaction must overcome the zeta potential.

Ziehl-Neelsen stain Stain often used in procedures for acid-fast staining. It is a carbolfuchsin method that involves the application of heat.

Ziemann dots Malarial pigment characterized as tiny, red-staining dots, smaller than Schüffner stippling, in red blood cells infected with *Plasmodium malariae*.

zone of equivalence Portion of a precipitin or agglutination curve when antibody and antigen concentrations are optimal for lattice formation.

zone of inhibition Zone related to disk diffusion testing; a clear area of no growth surrounding an antimicrobial disk following overnight incubation; results from diffusion of the antimicrobial molecules into the agar and growth inhibition of the test bacterium.

zoonosis (pl. zoonoses) Disease that humans acquire from exposure to infected animals or products made from infected animals.

zoonotic Pertains to diseases that can be transmitted from animals to humans.

Index

Page numbers followed by “f” indicate figures, “t” indicate tables, and “b” indicate boxes.

A

- A549 cells, 716–717
- Abbott Molecular, 948t
- Abdomen, examination of, 873
- Abiotrophia* spp., 338, 916
- Abscess fluid, mycotic sample collection of, 628
- Abscesses
 - brain, 892–893, 898–899, 899f
 - lung, 818–819
 - retroorbital, 800
 - specimen collection from, 125t–126t
- Acanthamoeba* spp., 656–657, 656f, 657f, 1011
- AccuProbe system, 231
- Accuracy, 116
- N-acetyl-d-glucosamine (NAG), 8, 9f, 252, 256f
- N-acetyl-d-muramic acid (NAM), 8, 9f, 252, 256f
- N-acetyl-L-cysteine (NALC), 586
- Achlorhydria, 871
- Achromobacter* spp., 488–489
- Achromobacter xylosoxidans*, 488, 489f
- Acid fastness, 571
- Acid-fast bacilli (AFB), 571, 580. *See also* *Mycobacterium* spp.
 - staining for, 586, 587t
- Acid-fast cell wall, 9
- Acid-fast stain, 13, 142–143, 143t
- Acidimetric test, 397
- Acinetobacter baumannii*, 485f
- Acinetobacter* spp., 484–485
 - clinical infections from, 484–485
 - identifying characteristics of, 485
- Acquired immunodeficiency syndrome (AIDS), 363, 740b, 943–944, 963b, 967b
 - acute infection of, 964
 - causes of, 963–964, 964f
 - clinical manifestations of, 964–966, 965b, 965t, 967t
 - clinically latent, 965
 - epidemiology of, 964
 - HIV progression to, 965–966, 966f
 - immunocompromised patients and, 979
 - immunologic markers of, 741b
 - laboratory diagnosis of, 966–967
 - overview of, 963–967
 - treatment for, 967, 967t
- Acquired resistance, 260
- Acridine orange stain, 12f, 13
- Actinomadura*, 362, 363t
- Actinomyces israelii*, 523f
 - Actinomyces* spp., 523
 - actinomycosis and, 503–504, 503f
 - Actinomycetes, aerobic, non-spore-forming, branching, 360–364
 - Actinomycosis, 503–504, 503f, 847, 847f
 - Actinomycotic mycetomas, 361, 608, 609f
 - Acute bacterial conjunctivitis, 1005–1006, 1005f
 - Acute care setting
 - frequently identified microbes in, 56, 56t
 - infection control in, 52t
 - Acute diarrhea, 872
 - Acute febrile respiratory illnesses, 809
 - Acute fungal sinusitis, 799
 - Acute glomerulonephritis, 329, 798
 - Acute hemorrhagic conjunctivitis, 1007f
 - Acute otitis media (AOM), 801
 - Acute pneumonia, 811–817
 - Acute respiratory disease (ARD), 719
 - Acute respiratory distress syndrome (ARDS), 810
 - Acute urethral syndrome, 925b, 932
 - Acute-phase serum specimens, 202
 - Adaptive immune response, 975
 - Adaptive immunity, 43–45
 - Additives, to urine, 934
 - Adenosine 5'-phosphosulfate (APS), 245
 - Adenosine diphosphoribose (ADPR), 349
 - Adenosine triphosphate (ATP), 245
 - Adenoviridae, 718–719
 - Adenovirus, 719, 719f, 809
 - CPE of, 717, 718f
 - Adenovirus serotype 14, 719
 - Adenovirus type 40, 719
 - Adenovirus type 41, 719
 - Adenylate cyclase toxin, 412
 - Adherence
 - mechanisms, of *Haemophilus influenzae*, 393
 - pathogenesis and, 35–37, 36f
 - virulence and, 35–37, 36f, 795
 - Adhesins, 35
 - Adhesion molecules, 710–712
 - Adults, UTI in, 925
 - Aedes aegypti*, 733
 - Aedes albopictus*, 733
 - Aerial mycelia, 598, 598f
 - Aerobes, 132–134
 - Aerobic diptheroids, 28
 - Aerobic gram-positive bacilli, 347, 348b, 349f
 - Aerobic respiration (oxidation), 17
 - Aerococcus* spp., 338
 - Aeromonas hydrophila*, 32–34, 465f
 - Aeromonas* spp., 455b–456b, 463–467, 463f
 - antimicrobial susceptibility for, 466–467, 467b
 - clinical manifestations of, 464
 - culture media of, 464–465, 465f
 - definitive identification of, 465–466, 466t
 - diarrhea and, 464
 - extraintestinal infection and, 464
 - general characteristics of, 463–464
 - intestinal infection and, 464
 - key features of, 458t
 - laboratory diagnosis of, 464–466
 - presumptive identification of, 465, 466f
 - skin infections from, 848
- Aerosols, 32
 - biological agents transmitted by, 757
- Aerotolerance test, 514, 514t
- Aerotolerant anaerobes, 15, 497t, 498
- Afipia felis*, 987
- African sleeping sickness, 664–665
- Agar dilution MIC test, 279, 279f
- Agar-based media, 587
- Agarose gel electrophoresis, 230, 233f
- Age
 - bacteremia and, 907, 907b
 - respiratory tract infections and, 794, 811t
 - urinary tract infections and, 925–927, 926f
- Agglutination, 200
- Agglutination assays, 206–210, 207f, 209f, 210f, 210t
- Aggregatibacter actinomycetemcomitans*, 399–401, 401f
- Aggregatibacter aphrophilus*, 399, 400f, 401f
- Aggregatibacter* species, 398t
- Aging, immunocompromised patients and, 981
- AIDS. *See* Acquired immunodeficiency syndrome
- Airborne transmission, 32
- Ajellomyces dermatitidis*, 612
- Alcaligenes faecalis*, 488–489, 489f
- Alcaligenes* spp., 488–489
- Alcoholic fermentation, 17
- Alcoholism, opportunistic microorganisms and, 31t
- Alcohols, 71–72, 71t
- Aldehydes, 72
- Alere Determine HIV-1/2Ag/Ab Combo assay, 966
- Alkaline phosphatase (AP), 212, 239
- Alphaviruses, 742–743, 857–858
- Alternaria* spp., 620, 621f
- Amantadine, 736
- Amastigote, 645

- Ambulatory care setting
frequently identified microbes in, 56
infection control in, 52*t*
- Amebae, intestinal, 646–647
life cycle of, 647–654, 648*f*
treatment of, 647
- American Thoracic Society (ATS) media, 587
- American Type Culture Collection (ATCC), 295–296
- Amherst, Jeffery, 754–755
- Amino acid utilization, 188–189, 189*f*
- Aminoglycosides, 253*t*–254*t*, 259, 290*t*
- δ-Aminolevulinic acid (ALA), 396–397
- Amoebae*, 1011
- Amorphous debris, 146, 147*f*
- Amphotericin B, 635
- Ampicillin, 277*t*
- Amplicon, 233
- Amplification-dependent methods, for
strain typing, 242–243
- AMV reverse transcriptase, 238–239
- Anabolism, 16
- Anaerobes, 132–134
antimicrobial susceptibility testing and, 527–529, 529*t*
options, 528–529
problems in, 528–529
biochemical-based identification systems and, 519
blood and, 506
of clinical importance, 496, 497*b*, 529*b*–530*b*
colony morphology and, 513–514, 513*f*
cultures, primary plating media for, 508–509, 508*t*, 513
defining, 497–498, 497*t*
definitive identification of, 518–521
direct microscopic examination of, 507
frequently encountered, 501–505
gastrointestinal tract and, 500
genitourinary tract and, 500
Gram stain reaction, 513–514, 514*f*
gram-negative, 504
clinical infections from, 504
identification of, 518, 519*f*, 519*t*, 520*f*, 524–526, 525*t*
gram-positive
identification of, 517–518, 518*b*, 518*f*, 518*t*, 523–524, 523*t*
non-spore-forming, 503–504
spore-forming, 501–503, 502*f*
human disease involvement indications of, 500–501, 501*b*
identification procedures for, 512–527, 512*t*
important concepts with, 497–501
indications of, 513–514
locations of, 498, 498*b*, 499*t*
macroscopic examination of, 507, 507*t*
media incubation and, 509, 509*f*
organisms as, 498
pathogens, recovery of, 506–512
predisposing factors with, 500, 500*t*, 501*b*
preliminary procedures for, 513
presumptive identification of, 514–518
respiratory tract and, 499–500
rRNA gene sequencing and, 521
- Anaerobes (*Continued*)
skin and, 499
at specific anatomic sites, 498–500, 499*t*
specimen selection, collection, transport, and processing of, 505–512, 505*b*, 505*t*
susceptibility testing and, 286–287
treatment for diseases associated with, 529–530, 529*b*
urease test of, 515
virulence factors of, 500*t*
- Anaerobic bags, 512, 512*f*
- Anaerobic blood agar, 171*t*, 508, 508*t*
- Anaerobic broth, 508*t*
- Anaerobic chambers, 506, 510–511, 510*f*
testing frequency of, 110*t*
- Anaerobic cocci, 504–505, 526–527, 527*f*, 528*t*
clinical infections from, 505
- Anaerobic jars, 511–512, 511*f*
- Anaerobic transport system, 127
- AnaeroPack System, 506
- Analyte-specific reagents (ASRs), 237–238
- Analytic analysis, of tests, 116
- Analytic (technical) sensitivity, 116
- Analytic (technical) specificity, 116
- Analytical activity, 104–105
- Analytical phase, of workflow, in
laboratory, 104–105, 104*f*
- Analytical profile index, 192–193
- Anamnestic immune response, 46, 201–202, 202*f*
- Anamorph, 600
- Anaplasma phagocytophilum*, 556
- Anaplasma* spp., 556
- Anaplasmataceae, 555–556
infection, 996–997, 996*b*
- Ancylostoma ceylanicum*, 695
- Ancylostoma duodenale*, 695
- Aneuploid, 716–717
- AN-IDENT, 520
- Animalcules, 4
- Annealing, primer, 232, 232*t*
- Annelids, 600
- Anneloconidia, 600
- Anorectal infections, 374–375
- Anotox, 510
- Anoxomat, 511, 511*f*
- Antagonism, 302, 303*f*
- Anthrax, 34*t*, 364, 755, 988–989, 988*b*
as bioterror agents, 755, 760–762
cause of, 988
clinical manifestations of, 988–989
cutaneous, 365, 760, 760*f*, 839, 988
epidemiology of, 988–989
gastrointestinal, 365, 760–761, 988–989
inhalation, 365, 760, 761*f*, 828, 989
injectional, 365–366
oral, 760–761
pulmonary, 989
treatment and management of, 989*t*
treatment of, 367
- Antibiograms, 296–297, 795
errors in, 298*t*
outbreak investigations and, 59, 59*t*
for several gram-negative species, 297*t*
- Antibiotics
cell wall biosynthesis inhibition and, 252–256, 255*f*
DNA replication interference of, 258
DNA transcription interference of, 258
folate synthesis inhibition and, 258, 258*f*
β-lactams, 253*t*–254*t*, 255, 266*t*
macrolide, 253*t*–254*t*, 259
mRNA translation interference of, 258–260
targets and mechanisms of action of, 252–260, 255*f*
- Antibodies, 40, 43–44
classification and characteristics of, 45–46
primary and secondary responses of, 46, 47*f*
in serologic testing, 201–205, 201*t*, 202*f*, 203*f*, 204*f*, 205*f*
- Antibody titers, 201–202
- Anticoagulants, 913–914
for bacteremia, 920
specimen and, 128
- Anticodon, 21
- Anticytokine therapies, for bacteremia, 920
- Anti-EA IgG, 723
- Anti-EA/D, 723–724
- Anti-EA/R, 724
- Anti-EBNA, 724
- Antifungal agents, 635
- Antifungal susceptibility testing, 635–636
- Antigen detection, for viral infections, 715
- Antigenic drift, 735
- Antigenic shift, 735, 808–809
- Antigens, 43–44, 200
direct antigen detection assays, 222–224, 222*t*
in Enterobacterales, 419
protective, 364
in *Salmonella*, 433, 434*f*
serologic testing, 201
in *Shigella*, 436
tolerance and, 47
Vibrio spp. and, 457–458
- Antimicrobial assays, 302
- Antimicrobial concentration test, 300*t*
- Antimicrobial pressure, 55
- Antimicrobial resistance
in Enterobacterales, 419–421
Enterococcus spp. and, 338
of *Neisseria gonorrhoeae*, 380
Streptococcus pneumoniae and, 334
- Antimicrobial stock solutions, 277
- Antimicrobial substances, as host resistance factors, 40–41
- Antimicrobial susceptibility testing. *See* Susceptibility testing
- Antimicrobials, 251*b*, 253*t*–254*t*, 529
for bacteremia, 919–920
combined mechanisms of action, 260
empiric therapy, 795
measurement of, 302–303
studying, biofilm models for, 787
susceptibility to, quality control and, 114–115, 115*t*
testing concerns, additional organism and, 287–291, 287*b*
- Antisepsis, 71, 920, 920*b*

- Antiseptics, 66–67
 disinfectants *versus*, 71–75
 drugs, 76t
- Antistreptolysin O test (ASO test), 220
- Antitoxins, 529
- Anti-VCA, 723
- Antiviral therapy, 749, 750t
- Antler hyphae, 598–599, 599f
- API 20NE, 478–481
- API Rapid Coryne System, 354
- Apicomplexa, opportunistic intestinal, 667–681
Babesia spp., 672–674
Plasmodium spp., 667–672. *See also Plasmodium* spp.
Toxoplasma gondii, 674–677
- Apocrine sweat glands, 28, 840–841
- Appliqué forms, 671–672
- APTIMA, 551–552
- Arboviruses, 728–729, 894, 898
- Arcanobacterium*, 358
- Arcanobacterium haemolyticum*, 358, 359f
- Archaea, 6
 classification by, 7
 gram-negative, 7
 gram-positive, 7
- Arcobacter*, 470t
- Arenaviridae, 729
- Arenavirus, 729
- Argentine hemorrhagic fever, 729
- Arterivirus*, 742–743
- Arthroconidium, 600, 600f
- Arthropod-borne diseases, 34
- Arthropods, 34, 992–997
- Arylsulfatase, 593
- Asaccharolytic bacteria, 185, 475
- Ascaris lumbricoides*, 693t, 695, 696f
- Ascending pyelonephritis, route of transmission, 33f
- Ascomycota, 600
- Ascospores, 600
- Ascus, 600
- Aseptic meningitis, 893
- Asexual reproduction, 667
 of fungi, 600, 600f
- Ashdown medium, 487
- Asian flu, 735
- Aspergillus* spp., 617–618, 617f, 618f, 843, 843t
 pneumonia and, 820
 respiratory tract infections and, 826
- Aspirates, 506, 506f
- Aspiration pneumonia, 806t–807t, 818–819
 case study on, 818b, 819b
 causes of, 818–819
 clinical manifestations of, 819
 complications with, 819
 diagnosis of, 819
 epidemiology of, 818
 pathogenesis of, 819
 treatment for, 819
- Asthma, 548
- Astroviruses, 875
- Asymptomatic bacteriuria, 936
- Asymptomatic gonococcal infection, 374
- ATP-binding cassette (ABC) superfamily, 262
- Attachment
 in biofilms, 781
 phagocytosis and, 41–42
- attI* gene, 268
- Atypical pneumonia, 408
- Aum Shinrikyo cult, 755
- Auramine stain, 586
- Auramine-rhodamine fluorochrome stain, 586
- Aureobasidium* spp., 621
- Autoclave, testing frequency of, 110t
- Automated identification systems
 for biochemical identification, 195–197, 195t
 evaluation of, 197
- Automated screening methods, for urine, 935, 936t
- Automated testing systems, 293–295
 BD Phoenix system, 293–294, 293f, 294f
 MicroScan WalkAway SI, 294–295, 294f
 Sensititre Automated Incubator Reader, 295
- AutoSCAN system, 196
- Autotrophs, 14
- Avian flu, 735
- Avian influenzas, 809
- Avibactam, 266
- Avidity, 203
- Axostyle, 661–662
- Azithromycin, 253t–254t
- B**
- Babès-Ernst granules, 350
- Babesia microti*, 672–673, 674f
- Babesia* spp., 672–674
 clinical infections of, 673
 laboratory diagnosis of, 674, 674f
 life cycle of, 673–674
- Babesiosis, 672
- Bacillary angiomatosis, 850
- Bacilli, 11, 11f
- Bacillus*, 364–368
 characteristics of, 364
- Bacillus anthracis*, 36t, 38t, 364–367, 755, 760–762, 762b, 988
 clinical infections of, 365–366
 clinical manifestations of, 760–761
 colonies of, 762f
 differentiation of, 367t
 direct examination of, 761
 isolation of, 135t
 laboratory diagnosis of, 366–367, 366f
 specimen collection and preparation of, 761
 virulence factors of, 364–365
- Bacillus cereus*, 32, 177, 367–368, 367t, 368f
- Bacillus cereus* biovar *anthracis*, 367
- Bacitracin susceptibility, 331t, 341, 343f
- Back safety, 97
- Background materials, 146–148, 147f
- BacT/ALERT 3D System, 916
- BACTEC 9000 Series, 915, 915f
- Bacteremia, 904, 904b–905b, 920b
 acceptable specimens for, 505t
 age of patient and, 907, 907b
 antimicrobial resistance and, 907–908
 antimicrobial therapy for, 919–920
 biomarkers for, 918–919, 919b
- Bacteremia (*Continued*)
 blood cultures for, 914–916
Brevundimonas spp. and, 489–490
 causative agent of, 906
 causes of, 908–909, 908t
 central nervous system infections and, 910
 classification of, 906
 clinical aspects of, 909–911
Clostridium perfringens and, 503
 community-acquired, 906
 continuous, 906
 definitions of, 905–906, 905t
 density of, in adults *versus* neonates, 911–912
 diagnostic criteria and coding practices for, 908
 duration of, 906
 epidemiology of, 906–908
 episodes of, *versus* time and frequency of collection, 912
 general concepts of, 905–906
 immunocompetency of patients and, 907
 incidence and mortality of, 906–907
 infective endocarditis and, 910
 intermittent, 906
 laboratory diagnosis of, 911–919
Lactobacillus spp. and, 504
 MALDI TOF for, 918, 919f
 multiple blood collections in, rationale for, 912–913, 913b
 musculoskeletal infections and, 910
 pathogenesis of, 909
 pneumonias, 910
 polymicrobial, 906
 prevention of, 920–921
 primary, 906
Pseudomonas aeruginosa and, 481
 risk factors for, 907–908
Salmonella causing, 435
 secondary, 906
 signs and symptoms of, 910–911
 site of origin of, 906
 skin infections and, 910
 specimen collection and, 911–913, 911b
 syndromes associated with, 909–910
 transient, 906
 unacceptable specimens for, 505b
 urinary tract infections and, 910
- Bacteria, 4
 assessment questions, 23b–24b
 cell structure of, 1, 3b, 4t–5t, 5f, 9b, 23b
 classification/taxonomy of, 6–7
 genetics of, 1, 3b, 4t–5t, 5f, 9b, 23b
 terminology, 21
 growth of, 13–16
 metabolism of, 1, 3b, 4t–5t, 5f, 9b, 23b
 morphology of, 11–13
 microscopic shapes, 11, 11f
 physiology of, 1, 3b, 4t–5t, 5f, 9b, 23b
- Bacterial blepharitis, 1008
- Bacterial endophthalmitis, 1014
- Bacterial genome, 21
- Bacterial keratitis, 1009f, 1010b
- Bacterial meningitis, 891–893, 891b, 892f, 893f
- Bacterial pathogens, diarrhea and, 875–879, 875b
- Bacterial pharyngitis, *Streptococcus pyogenes* and, 328, 328b
- Bacterial tracheitis, 393

- Bacterial vaginosis (BV), 359, 504
 clinical manifestations of, 950
 epidemiology of, 950
 laboratory diagnosis of, 950, 950f
 overview of, 950–951
 treatment of, 951, 951b
- Bacteriocins, 28
- Bacteriophage, 6
- Bacteriuria, 923, 925b
- Bacteroides* bile esculin agar (BBE), 508, 508t, 519f
- Bacteroides fragilis*, 498, 524
- Bacteroides* spp., 524
- Bacteroides thetaiotaomicron*, 524, 526f
- Bacteroides ureolyticus*, 518
- BACTiStaph test, 318, 320f
- Balamuthia mandrillaris*, 657
- Balance, for weights, testing frequency of, 110t
- Balaneatrix*, 490
- Balantidium coli*, 657, 657f
- Bang disease, 990
- Barriers, physical, 39
- Bartlett's Q scoring of sputum samples, 149, 149t
- Bartonella* spp., from blood culture, 917
- Bartonellosis, 850
- Baseline data, 54
- Basidiomycota, 601
- Batch processing, 509–510
- Bats, 731
- Bayes's theorem, 117
- BBL Crystal anaerobe identification system, 520
- BBL Crystal GP System, 354
- BBL Staphyloslide test, 318
- BD Diagnostic Systems, 948t
- BD FX, 915
- BD Phoenix Automated Microbiology System, 195t, 197
- Beauveria bassiana*, 618, 618f
- Becton-Dickinson Diagnostic Systems, 506
- Benchmarking, 109–110
 operations, 109–110
 quality, 110
- Benzalkonium chloride, 586
- Bergeyella* spp., 490
- Bias, 116
- Bifidobacterium* spp., 523
- Bile disk, 516
- Bile esculin test, 330, 331t, 343, 343f
- Bile solubility test, 194t, 344
- Bilharziasis, 685
- Bilophila* spp., 524
- Bilophila wadsworthia*, 518, 524
- Biochemical identification, 183, 184b, 191b, 192b
 amino acid utilization, 188–189, 189f
 assessment questions, 197–198
 carbohydrate utilization and, 184–187, 184b, 185f, 186t, 194, 194t, 195f
 evaluation of, 197
 glucose metabolism and, 187–188
 manual multitest systems for, 192–193, 192t
 miscellaneous tests for, 189–192, 190f
 of *Mycobacterium* spp., 590–593, 592f, 593f
 points to remember, 197
 rapid and automated identification systems for, 193–197, 194t, 195t
- Biochemical-based identification systems, anaerobes and, 519
- Biochemistry, bacterial, 16–19
- Biocrime, 755, 755b
- Biofilm phenotype, 778
- Biofilms, 776, 776b–777b, 777f
 antimicrobial studies with models of, 787
 architecture of, 780, 780t
 in attachment, 781
 chronic upper respiratory tract infections due to, 784
 as community of cells, 778, 778f
 cystic fibrosis, 784
 as defense mechanism, 781–782
 definition of, 777b
 dental, 782–784
 detection of, 786
 disaggregation and, 782, 782b
 diseases associated with, 782–785, 783t
 formation of, 778–780, 779t
 gene transfer and expression, 782
 IMDs and, 784–785, 785t
 laboratory consequences associated with, 785–786
 metabolism benefits of, 781
 ocular infections and, 1014–1016, 1015t, 1016f
 pathogenicity of, 780–782, 781b
 potential intervention for, 786–788
 properties of, 780
 resistance and, 261
 sterilization and disinfection and, 68
- Biofouling, 777
- Biohazard hood, testing frequency of, 110t
- Biolog database, 197
- Biolog Microbial ID System, 478–481
- Biolog OmniLog ID System, 197
- Biological agents, 756
 general characteristics of, 757–759, 757b, 758t
 select, 404, 757
- Biological assays, 302–303
- Biological risk assessment, 79–81, 759
- Biological safety cabinet (BSC), 82, 83b, 83f, 583
- Biological warfare, 754
- Biomarkers, for bacteremia, 918–919, 919b
- Biopsy specimens, 645
- Biorisk, 758
- Biorisk management, 758–759
- Biosafety, 759
- Biosafety in Microbiological and Biomedical Research Laboratories* (BMBL), 759
- Biosafety levels (BSLs), 81–85, 759–760, 759t, 760b
 level 1, 82–84
 level 2, 84
 level 3, 84
 level 4, 84–85
- Biota
 resident, 76
 transient, 76
- Bioterror agents, 753, 754b, 773b
Bacillus anthracis, 760–762
 biological risk management of, 758–759
 botulinum as, 764
Burkholderia spp. as, 762–764
Francisella tularensis as, 766–767, 767f
- Bioterror agents (*Continued*)
 general characteristics of, 757–759, 757b, 758t
 key indicators of, 755b
 laboratory response network and, 756–757, 756b, 756f, 757f
 Poxviridae as, 767
Salmonella as, 755
 smallpox as, 727, 727f
 toxins as, 771
 variola virus as, 767–769, 768f
Yersinia pestis as, 769–771
- Bioterrorism, 286, 754
 readiness for, 771–772
 respiratory tract infections and, 827–829
 safety during, 98–100
- Biothreat agents, 756–757
- Biotin, 215
- Biovarieties, 7
- Biphasic system, 626–628
- Bipolar staining, 404, 437
- Bites, 841
 in diseases, to humans and horses, 742–743
 transmission by, 34
- Black death, 754, 992
- Black dot ringworm, 605
- Black eschar, 365, 760
- Black piedra, 602–603
- Blackwater fever, 668
- Bladder catheterization, 927
- Blastoconidium, 598, 598f, 633–634
- Blastocystis* spp., 650t, 652–654, 654f
- Blastomyces dermatitidis*, 612, 612f, 852, 852f
- Blastomyces gilchristii*, 612
- Blastomyces* spp., 600, 610–612, 612f
- Blastomycosis, 610–612
 chromoblastomycosis, 844f
 tuberculosis and, 820
- Blepharitis, 1002
 bacterial, 1008
 viral, 1008–1009
- Blood
 anaerobes and, 506
 flagellates, 662–667, 663f
 flukes, 685–687
 clinical infections of, 685–686
 laboratory diagnosis of, 686–687, 687f
 life cycle of, 686, 686f
Mycobacterium spp. and, 585
 parasites and, examination of specimens for, 644–645
 rationale for multiple collections of, bacteremia and, 912–913, 913b
 roundworm infections in, 700–706
- Blood cultures
 bacteremia and, 911–913
Bartonella spp. recovery in, 917
Brucella spp. recovery in, 916
Campylobacter spp. recovery in, 916
 contamination in, 917
 continuous-monitoring, 915–916
Coxiella burnetii recovery in, 916
Francisella tularensis recovery in, 916
 HAECK group of gram-negative bacilli recovery in, 917
Leptospira spp. recovery in, 916
 mycobacteria from, 917

- Blood cultures (*Continued*)
 nutritionally variant streptococci from, 916
 rapid identification of microorganisms growing in, 917–918
 recovery of organisms from, 916–917
 systems for, 914–916
 specimen collection from, 125t–126t
- Blood smears, 644–645
- Bloodborne pathogens, 78, 745
- Bloodstream infection, 53t
- Body fluids, specimen collection from, 125t–126t
- Bone marrow, 626–628
- Borderline oxacillin-resistant isolates, 288
- Borderline oxacillin-resistant *Staphylococcus aureus* (BORSA), 321
- Bordetella*, 411–416
 antimicrobial susceptibility of, 414–416
 clinically significant species of, 412
 colony morphology of, 413, 413f
 general characteristics of, 412
 identification of, 413, 414t
 isolation of, 413
 laboratory diagnosis of, 412
 serologic testing of, 413–414
 specimen collection and handling of, 413
- Bordetella parapertussis*, 412
- Bordetella pertussis*, 38t, 412
 isolation of, 135t
- Bordet-Gengou potato infusion agar, 413
- Borrelia burgdorferi*, 537, 993
- Borrelia recurrentis*, 536–537, 536f
- Borrelia* spp., 536, 536f
- Borreliosis, 848
- Botulinum toxin, 755, 764
- Botulism, 502
- Bouquet, Henry, 754–755
- Boutonneuse fever (BF), 553, 850, 995
- Bradyzoites, 675
- Brain abscess, 892–893, 898–899, 899f
- Branched DNA detection (bDNA detection), 239, 240f
- Brazilian purpuric fever, 394
- Break bone fever, 733
- Breakpoint (cutoff) MIC panels, 278
- Brevundimonas* spp., 489–490
- Bridge bacteria, 783
- Brill-Zinsser disease, 553, 995
- Brincidofovir, 727
- Brittle consistency, of colony, 176
- Broad-spectrum activity drug, 76t
- Bronchial lavage fluid, 549
- Bronchitis and bronchiolitis, 805–808, 806t–807t
 causes of, 805–807
 clinical manifestations of, 807–808
 complications with, 808
 epidemiology of, 805
 laboratory diagnosis of, 808
 pathogenesis of, 807
 treatment for, 808
- Broth macrodilution MIC tests, 277, 300f
- Broth media, 14, 131
- Broth microdilution MIC tests, 277–279, 277f, 278f, 278t, 293
- Broth systems, conventional, culture media used in, 913–914
- Brown-Brenn stain, 1012
- Brown-Hopps stain, 1012
- Bruce, David, 990
- Brucella abortus*, 36t
- Brucella* blood agar (BRU/BA), 508t, 524–526
- Brucella* spp., 404–406, 406f, 406t
 from blood culture, 916
 isolation of, 135t
- Brucellosis, 34t, 404, 990
- Brugia malayi*, 701, 702t
- Buboes, 394, 437, 992
Chlamydia trachomatis and, 545
Yersinia and, 769–770, 770f
- Bubonic plague, 437, 769, 992
- Budvicia*, 439
- Buffer, 232, 233f
- Buffered charcoal yeast extract (BCYE) agar, 409, 410f
- Bullae, 835b, 837
- Bullous impetigo, 311
- Bundibugyo ebolavirus (BEBOV), 765
- Bunyavirales, 729–730
- Burkholderia cepacia* complex, 486
- Burkholderia gladioli*, 487–488
- Burkholderia mallei*, 487
- Burkholderia pseudomallei*, 487, 487f
- Burkholderia* spp., 486–488, 486f, 762–764
 clinical manifestations of, 763
 direct examination of, 763, 763f
 specimen collection and preparation of, 763
- Burkitt lymphoma, 723
- Burns, 979
 opportunistic microorganisms and, 31t
- Butanediol fermentation, 17
- Buttiauxella*, 439
- Butyric acid fermentation, 17
- Butyrous consistency, of colony, 176
- C**
- Cadaverine, 188–189
- Calabar swellings, 701–702
- Calcofluor white stain, 13, 142–143, 143t, 625–626
- Caliciviridae, 730–731, 875
- Caliciviruses, 875
- California encephalitis virus, 729
- CAMP test. *See* Christie, Atkins, Munch-Peterson (CAMP) test
- Campylobacter blood agar (Campy-BA), 171t
- Campylobacter jejuni*, 15, 32–34, 876, 884, 884f
- Campylobacter* spp., 455b–456b, 467–471
 antimicrobial susceptibility for, 471
 from blood culture, 916
 clinical manifestation of, 468
 clinically significant species of, 467t
 colony morphology of, 469–470
 culture media of, 468–469, 469t
 definitive identification of, 470–471, 470t, 471f
 epidemiology of, 467–468
 immunologic assays for, 471
 incubation of, 469
 laboratory diagnosis of, 468–471
 microscopic morphology of, 469, 469f
- Campylobacter* spp. (*Continued*)
 presumptive identification of, 469–470
 specimen collection and transport of, 468
- Campylobacter ureolyticus* group, 526
- Campylobacter*-like species, 467–471
- CA-MRSA. *See* Community-associated methicillin-resistant *Staphylococcus aureus*
- Canaliculitis, 1013
- Cancer patients, 977–978
- Candida albicans*, 181f, 622
- Candida dubliniensis*, 632
- Candida* spp., 622–623, 623f, 834–835, 950, 953f
- Candidiasis
 clinical manifestations of, 952–953, 953f
 epidemiology of, 952
 laboratory diagnosis of, 953
 overview of, 952–953
 skin lesions, 852
 superficial, 834–836
 treatment of, 953, 953b
 vulvovaginal, 952
- Candle extinction jar, 376
- CAP. *See* College of American Pathologists
- Capnocytophaga*, 403–404, 403f, 404f
- Capnocytophaga canimorsus* infection, 987, 987b
- Capnophiles, 132–134
- Capnophilic, defined, 325, 373
- Capnophilic organisms, 15, 497, 497t
 HACEK group as, 399
- Capsid, 710
- Capsomers, 712–713
- Capsule
 bacteria, 5f, 10
Haemophilus influenzae and, 392
 resistance to phagocytosis with, 35
 of streptococcal cell wall, 326f
- Carbapenemase-producing Enterobacterales (CPE), 291
- Carbapenemases, 291
- Carbapenems, 255, 256t
- Carbohydrate assimilation, yeast and, 632–633
- Carbohydrate chains, plasma membrane, 8f
- Carbohydrate utilization
 lactose fermentation and, 19
 for *Neisseria gonorrhoeae*, 378
- Carbohydrates
 biochemical identification for, 184–187, 184b, 185f, 186t, 194, 194t, 195f
 fermentation and, 185
 gram-negative bacteria and, 184–187, 184b, 185f, 186t, 194, 194t, 195f
 group, of streptococcal cell wall, 326f
 nonfermenting gram-negative bacilli and, 475
- Carbon dioxide, 510–511
- Carbon dioxide incubator, 15
- Carbuncles, 310, 840
- Cardiobacterium hominis*, 401–402, 402f
- Cardiovascular disease, 548
- Carrier, 27
- Carrier state, 27, 435
- Cary-Blair transport medium, 128
- Cases, infection control and, 55
- Caspofungin, 635

- Cat bites, 841
- Cat scratch disease (CSD), 850, 987–988, 988b
- Cat tapeworm, 692
- Catabolism, 16, 16f
- Catalase, 498
- Catalase test, 194t, 515, 592, 592f
- Catalase-negative bacilli, non-spore-forming, nonbranching, 356–360
- Catalase-positive bacilli
non-spore-forming, nonbranching, 348–356
spore-forming, nonbranching, 364–368
- Catarrhal phase, of pertussis, 412
- Category B select biological agents, 404
- Catheter, specimen collection from, 125t–126t
- Catheter tips, specimen collection from, 125t–126t
- Catheter-associated urinary tract infections (CAUTIs), 52–54, 53t
- Catheterized specimen collection, 933, 937
- Catheter-related bloodstream infection, bacteremia and, 909–910
- CAUTIs. *See* Catheter-associated urinary tract infections
- CD4 cell count, 823, 824t
- CDC Biofilm Reactor, 787
- CDC Groups EO-3, EO-4, 490
- Cedecea*, 439
- Cefinase disks, 291–292
- Cefsulodin-irgasan-novobiocin (CIN) agar, 438
- Cell appendages, of prokaryotes, 10
- Cell culture, 713, 716–717, 717t
cytopathic effect on, 717–718
- Cell envelope, 255f
- Cell membrane, of streptococcal cell wall, 326f
- Cell numbers, determination of, 16
- Cell wall
absence of, 9
acid-fast, 9
antibiotic inhibition of biosynthesis of, 252–256
archaeal, 7
of eukaryotes, 11
gram-negative, 9, 9b, 252, 255f
gram-positive, 8, 9b, 252, 255f
of prokaryotes, 8–10, 9f
Streptococcus spp. structure of, 325–326, 326f
- Cell wall-deficient bacteria, 559
- Cell-bound coagulase, 316
- Cell-mediated immune (CMI) response, 580
- Cellophane tape preparation, 631
- Cellular fatty acid, 520
analysis, 520
- Cellular immune response, 977
- Cellulitis, 328, 838t, 839–840
- Erysipelas, 838–839, 838t, 839f
- Orbital, sinusitis and, 800
- Recurrent, 839
- Centers for Disease Control and Prevention (CDC), 547, 575, 727, 944, 976
- Centor criteria, 797b
- Central line-associated bloodstream infections (CLABSI), 52, 53t
- Central nervous system, 890
anatomic organization in, 890–891, 890f
bacteria involving, 891b
cerebrospinal fluid characteristics and, 891
host-pathogen relationship and, 891
infections of, 889, 889b–890b, 902b, 903b.
See also Meningitis
bacteremia and, 910
bacterial pathogens and, 899, 901
brain abscess and, 892–893, 898–899, 899b, 899f
fungal infections and, 895–896, 896b, 896f, 902
fungal pathogens and, 899
general concepts related to, 890–891
helminths and, 897–898
laboratory diagnosis of, 899–903, 900f
meningoencephalitis and encephalitis, 898–899
mycobacterial infections and, 895
parasitic infections and, 896–898, 902–903, 902b, 902f
protozoa and, 896–897, 897f
shunt infections in, 893
spirochetal infections and, 895, 902
viral agents involving, 894b
- Central spores, 501, 502f
- Centrifugation-enhanced shell vial culture, 718
- Centrifuge, testing frequency of, 110t
- Centrioles, 5f
- Centrosome, 5f
- Cephalomycins (CMYs), 266
- Cephalosporins, 256t
- Cephems, 255, 256t
- Cercaria, 682
- Cerebral malaria, 897
- Cerebrospinal fluid (CSF), 890, 897b
characteristics of, 891
fungal infections and, 895, 896f
mycotic sample collection of, 628
parasites and, 645, 897, 897f
shunt infections and, 893
transport and analysis of, 899–900
- Cervarix, 726, 968
- Cervical HPV lesions, 726
- Cervicitis, 925b, 931, 948–949
causes of, 948–949
clinical manifestations of, 949, 949b
epidemiology of, 949
laboratory diagnosis of, 948t, 949
overview of, 948–949
treatment of, 949
- Cestodes, tissue infections with, 687f, 691–692
- Chaetomium* spp., 621, 621f
- Chagas disease, 666
- Chancre, 539, 957, 957f
- Chancroid, 391, 394f
buboes and, 961
clinical manifestations of, 959, 960f
epidemiology of, 959, 960f
laboratory diagnosis of, 959–960, 960f
overview of, 959–960
treatment of, 960
- Chemical methods, of disinfection and sterilization, 70, 71t
- Chemicals
hazardous, 95b
classification of, 95–96
laboratory safety for, 96
inventory of, 95
spills, 96
storage of, 95
- Chemiluminescent immunoassay (CLIA), 219
- Chemosterilizers, 70
- Chemotactic agent, 41
- Chemotaxis, 41, 41f
- Chicago disease, 612
- Chickenpox, route of transmission, 33f
- Children, UTI in, 925
- Chilomastix mesnili*, 662, 662f
- Chinese liver fluke, 684
- Chlamydia, 943–944
FDA-approved molecular tests for, 948t
ocular infections, 1006–1007, 1006f
reported cases by, 945, 946f
- Chlamydia pneumoniae*, 547–548, 548t
- Chlamydia psittaci*, 546t, 548–552
antibody detection of, 552
cell culture for, 551, 552f
direct microscopic examination of, 549–551, 551f, 552f
immunoassays for, 551
laboratory diagnosis of, 549–552, 549t, 550t
for NAATs, 551–552, 552b
- Chlamydia* spp., 543b–544b, 544–552, 545t
comparative properties of, 545t
general characteristics of, 544
human diseases caused by, 546t
life cycle of, 544, 546f
newborn infection with, 547, 547b
- Chlamydia trachomatis*, 544–547, 546t, 1003
bubo formation and, 545
clinical infections of, 545–547
direct detection of, in urethral and cervical specimens, 118, 118t
elementary bodies and cells in, 544, 546f
gonorrhea from, 374
neonates and, 547, 547t
newborn infection with, 547, 547t
- Chloramphenicol, 253t–254t
- Chlorhexidine gluconate (CHG), 73
- Chlorine, 72–73
- Chloroplasts, 10
- Chloroquine, for malaria, 668
- Chloroxylonol, 74
- Chocolate (CHOC) agar, 171t, 172f, 174f, 914
for *Haemophilus*, 394–395, 395f
for *Neisseria gonorrhoeae*, 375–376
- Cholera, 456, 458, 877
- Cholera toxin, 458, 459f
- Cholera toxin, 458
- Cholesterol, in plasma membrane, 8f, 11
- Christensen's urea medium, 470
- Christie, Atkins, Munch-Peterson (CAMP) test, 311, 330, 331t, 332f, 341, 343f
- CHROMagar, 440, 442f
- Chromatographic assays, 303
- Chromatography, *Mycobacterium* spp. and, 593

- Chromatoidal bars, 643
Chromobacterium spp., 490
Chromobacterium violaceum, 176, 490
 Chromoblastomycosis, 607, 607t, 608f, 844f
 Chromogenic cephalosporin nitrocefin, 291–292
 Chromogenic cephalosporin test, 397
 Chromogenic substrates, 193–194, 378–380, 378t, 633
 Chromomycosis, 607
 Chromosomal DNA, restriction enzyme analysis of, 242
 Chromosomal mutation, 264
 Chromosomally mediated resistant *Neisseria gonorrhoeae* (CMRNG), 286
 Chromosomes, bacteria, 5f, 10
 Chronic ambulatory peritoneal dialysis (CAPD), 484
 Chronic bacterial conjunctivitis, 1006
 Chronic diarrhea, 872
 Chronic granulomatous disease (CGD), 486
 Chronic pneumonia, 806t–807t, 817, 820b
 Chronic upper respiratory tract infections, 784
Chryseobacterium indologenes, 491, 491f
Chryseobacterium spp., 490
Chrysosporium spp., 619, 619f
 Cidofovir, 727
 Ciguatera, 881
 Cilia, 5f
 Ciliates, 657–658
 Ciprofloxacin, 253t–254t
 Citrate utilization test, 189, 190f
Citrobacter, 428–429, 428b
Citrobacter freundii, 428
Citrobacter koseri, 428–429, 428b
 CLABSIs. *See* Central line-associated bloodstream infections
Cladophialophora carrionii, 607t
Cladosporium spp., 621, 621f
 Clavulanic acid, 263
 Clean-catch midstream urine specimen, 125t–126t, 126
 Cleansing mechanism, 39–40
 Clearview Complete HIV-1/2, 741
 Clearview HIV-1/2, 741
 CLIA. *See* Clinical Laboratory Improvement Act
 Clindamycin, 253t–254t, 289, 322
 Clinical analysis, of tests, 116–117
 Clinical and Laboratory Standards Institute (CLSI), 111, 272–273, 397, 463, 513
 Clinical Laboratory Improvement Act (CLIA), 106, 713
 Clinical (diagnostic) sensitivity, 116–117
 Clinical (diagnostic) specificity, 117
 Clinical virology, 708, 750b
 case on, 709b
 issues to consider, 709
Clonorchis sinensis, 684, 685f
 Close contact, 34
Clostridioides difficile, 38t, 503, 522, 878, 886
Clostridioides difficile-associated disease (CDAD), 503, 508
Clostridium botulinum, 32, 38t
Clostridium botulinum neurotoxin, 764
Clostridium novyi, 38t
Clostridium perfringens, 36t, 38t, 500
Clostridium septicum, 38t
Clostridium sordellii, 38t, 522
Clostridium spp.
 clinical infections, 501
 identification of, 521–523, 521f, 522t
Clostridium tetani, 8, 38t
 CLOtest Rapid Urease Test, 471
 CLSI. *See* Clinical and Laboratory Standards Institute
 Clue cells, 359
 Clumping factor, 309
 Cluster, 56–57
 Coagulase, 309
 Coagulase-negative staphylococci (CoNS), 309
 urinary tract infections caused by, 931t
 Coagulase-positive staphylococci, 309, 318t
 Coagulase-reacting factor (CRF), 316
 Coagulase test, 309
 Cobiofilms, 777, 777f
 Cocci, 11, 11f
Coccidioides immitis, 600, 896
 as opportunistic pathogens, 820
Coccidioides posadasii, 600
Coccidioides spp., 612–613, 613f
 Cochleates, 269
 Codon, 21
 Cold enrichment, 355–356
 Colistin, 487
 Colistin susceptibility, 384
 Colistin-nalidixic acid (CNA), 508, 508t
 Colitis, 782
 College of American Pathologists (CAP), 110
 Colon, normal microbiota of, 29
 Colonization, 26, 793
 Colony counts, historical background of, 931–932
 Colony morphology
 anaerobes and, 513–514, 513f
 Bordetella and, 413, 413f
 of *Campylobacter* species, 469–470
 of Enterobacterales, 418
 Haemophilus and, 395
 Legionella and, 409–410, 410f
 Mycobacterium spp. and, 589
 of *Neisseria gonorrhoeae*, 376–377, 376f, 377f
 Neisseria spp. and, 385t
 for presumptive identification of microorganisms, 169b–170b
 assessment questions, 181–182
 color and, 176, 176f
 consistency and, 176
 culture media and, 170–173
 density and, 175, 175f
 as diagnostic tool, 170
 elevation and, 175, 175f
 form of margin and, 174–175
 gross colony characteristics and, 173–177
 hemolysis and, 173–174
 initial observation and, 170–173
 liquid media growth and, 177–178
 multiple characteristics and, 177, 177f
 Colony morphology (Continued)
 odor and, 177
 pigment and, 176
 points to remember, 181
 size and, 174, 174f
 of *Streptococcus pneumoniae*, 179f
 Colony-forming units (CFU), 144–146, 273
 Color, colony morphology and, 176, 176f
 Colorado tick fever virus, 728–729
 Columbia colistin-nalidixic acid (CNA) agar, 171t
 Columbus, Christopher, 539
Comamonas spp., 490
 Combo trays, 196
 Commensal *Neisseria* species, 384–388, 384t, 386t
 Commensalism, 26
 Committees and programs, laboratory reporting to, 61
 Communal living settings
 frequently identified microbes in, 56, 56t
 infection control in, 52, 52t
 Community-acquired infections, 52
 Community settings
 frequently identified microbes in, 55–56
 infection control in, 52t
 Community trends, in respiratory tract infections, 795
 Community-acquired bacteremia, 906
 Community-acquired pneumonia, 806t–807t, 811–815
 causes of, 811–812
 clinical manifestations of, 812–813, 812f
 complications of, 813
 epidemiology of, 811
 laboratory diagnosis of, 813–814, 813f, 814f
 pathogenesis of, 812
 treatment for, 814–815
 Community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA), 320
 Competency, personnel, assessment of, 105–106, 107f
 Competent cells, 22
 Complement, 43–44
 Complement deficiency, 979
 Complement fixation (CF), 200, 218f
 Complement fixation test, 217–218
 Complement system, 40, 979
 Compressed gases, storage of, 97
 Concentration techniques, 642–643
 Condylomata accuminata, 967
 Condylomata lata, 957–958, 957f
 Confocal laser scanning microscopy (CLSM), 786
 Congenital infections, 722, 981–982
 Congenital syphilis, 539, 958
 Conidiogenous cells, 600
 Conidium, 600
 Conjugates, 210
 Conjugation, 21, 22f, 23, 267
 pili, 10
 Conjunctivitis, 782, 1002
 acute bacterial, 1005–1006, 1005f
 acute hemorrhagic, 1007f
 acute viral, 1007–1008

- Conjunctivitis (*Continued*)
 bacteria, 1005–1007
 chlamydial ocular infections and, 1006–1007, 1006f
 chronic bacterial, 1006
 fungi and, 1008
 microorganisms associated with, 1005b
 overview of, 1004–1005
 parasites and, 1008
 Parinaud oculoglandular, 545
 viral, 1007–1008
 Consistency, colony morphology and, 176
 Contact lenses and solutions, 1019f, 1020, 1020b
 Contact time, sterilization and disinfection and, 68
 Contaminated ocular medications, 1020, 1020b
 Contaminating materials, direct examination of, 155, 155f, 156f
 Contamination
 in blood cultures, 917
 microscopic examinations and, 149–150, 149f
 by normal microbiota, 146
 ocular medications and, 1020, 1020b
 PCR prevention of, 233
 Continuing education (CE), 106
 Continuous bacteremia, 906
 Continuous cell cultures, 716–717
 Continuous quality improvement (CQI), 105
 Controlling test variables, of MBC tests, 301
 Convalescent phase, of pertussis, 412
 Convalescent-phase serum specimens, 202
 Core window, of hepatitis B virus, 745
 Cornea infections, 1009–1012
 Cornea storage media, 1021–1022
 Cornmeal agar, 633–634, 634f
 Coronaviridae, 731–732
 Coronavirus disease 2019 (COVID-19), 21, 875
 Coronaviruses (CoVs), 731, 731f, 732b
Corynebacterium, 348–351
 characteristics of, 359t
 identification of, 354
Corynebacterium amycolatum, 351, 352t
Corynebacterium diphtheriae, 38t, 349–351, 352t
 clinical infections of, 349–350
 culture characteristics of, 350, 351f
 identification of, 351, 352t, 353f
 isolation of, 135t
 laboratory diagnosis of, 350–351
 microscopy of, 350, 350f
 toxigenicity of, 351
 virulence factors of, 349
Corynebacterium jeikeium, 351–354, 352t
Corynebacterium pseudodiphtheriticum, 352t, 353
Corynebacterium pseudotuberculosis, 352t, 353
Corynebacterium striatum, 352t, 353
Corynebacterium ulcerans, 352t, 353–354
Corynebacterium urealyticum, 352t, 354
 Coryza, 804
 COVID-19, 810–811
 Cowdry type A bodies, 715
Coxiella burnetii, 556
 from blood culture, 916
Coxiella spp., 556
 Coxsackieviruses, 739, 854
 CQI. *See* Continuous quality improvement
 Creamy consistency, of colony, 176
 Creutzfeldt-Jakob disease (CJD), 749
 CRF. *See* Coagulase-reacting factor
 Crick, Francis, 19
 Crimean-Congo hemorrhagic fever (CCHF) virus, 729
 Critical materials, disinfection and sterilization and, 69, 69t
 Critical values, 137b, 910b
 Crohn disease, 853
Cronobacter, 427
Cronobacter sakazakii, 427, 428f
 Cross-functional teams, 109
 Cross-reactivity, antibodies and, 204
 Cryptococcal antigen, 635
 Cryptococcal Antigen Latex Agglutination System (CALAS), 224
Cryptococcus neoformans, 36t, 895, 896f
Cryptococcus spp., 623, 624f
 pneumonia and, 820
 Cryptosporidiosis, direct antigen detection assay for, 223–224
Cryptosporidium hominis, 677, 880
Cryptosporidium parvum, 677, 880
Cryptosporidium spp., 677
 clinical infections of, 677
 laboratory diagnosis of, 678–679, 679f
 life cycles of, 678, 678f
 Crystal enteric nonfermenter system, 478–481
 CSF. *See* Cerebrospinal fluid
 CTL. *See* Cytotoxic T lymphocytes
 Culture confirmation probe applications, 231
 Culture media, 630, 631t
 selection of, 131–132, 133t
 types of, 131
 Cultures
 actively growing, laboratory safety and, 82, 82b
 of *Bacillus anthracis*, 761, 762f
 blood, bacteremia and, 911–913
 of *Burkholderia* spp., 763, 763f
 cell, 713, 716–717, 717t
 colony morphology and, 170–173
 of Enterobacterales, 440
 of *Francisella tularensis*, 766
 ocular infections, 1017–1019, 1018t, 1020f
 organ, 716
 outbreak investigations and, 59–60
 review of, data gathering and, 54–55
 shell vial, 718
 surveillance, 54
 tissue, 716
 for urinary tract infections, 936, 938f, 939t
 workup, 134–136
 of *Yersinia pestis*, 770–771, 771f
 Cumulative antibiograms, 297–298
Cunninghamella spp., 616, 616f
Cupriavidus pauculus, 492
Cupriavidus spp., 492
 Curschmann's spiral, 147, 147f
Curvularia spp., 621–622, 621f
 Customer concept, 109
 Cutaneous anthrax, 365, 760, 760f, 988
 Cutaneous diphtheria, 349–350
 Cutaneous infections, *Nocardia* causing, 361
 Cutaneous mycoses, 602, 602t
 agents of, 604–607
Cutibacterium acnes, 28
 Cuts, transmission by, 34
 Cyclic adenosine monophosphate (cAMP), 422
 Cycloserine-cefoxitin-fructose agar (CCFA), 522
Cyclospora cayentanensis, 679–681, 680f, 880
 Cystic fibrosis, 980–981
 biofilm, 784
 opportunistic microorganisms and, 31f
 Cysticercosis, 691–692
 Cysticercus, 687–688
 Cystine trypticase agar (CTA), 378
 Cystine-tellurite blood agar (CTBA), 350, 353f
 Cystitis, 925b, 931
Cystoisospora belli, 679, 680f, 880
 Cysts, 641–642
 Cyto centrifugation, 141–142, 142f
 Cytokine storm syndrome, 732
 Cytolytic toxins, 310
 Cytomegalovirus (CMV), 717, 718f, 722, 722f, 723f
 cancer patients and, 978
 diarrhea and, 882
 skin infections and, 856
 Cytopathic effect (CPE), 140, 717–718
 HSV and, 717f, 955
 neutralization assays and, 210
 of viruses, 715
 Cytoplasmic membrane, bacteria, 5f
 Cytoplasmic structure
 of eukaryotes, 10–11
 of prokaryotes, 7–8
 Cytopyge, 657–658
 Cytostome, 657–658, 657f
 Cytotoxic T lymphocytes (CTLs), 44–45, 47
- ## D
- Dacron, 713
 Dacryoadenitis, 1013
 Dacryocystitis, 1013, 1013f
 Dalbavancin, 253t–254t
 Dalfopristin, 259–260
 Dandruff, 603
 Daptomycin, 253t–254t, 787
 “Darting” motility, 469
 Data gathering, surveillance and cases, 55
 culture review, 54–55
 in infection control, 54–55
 laboratory support, 55
 Data mining, 54–55
 Deaminase test, 189
 Decarboxylase test, 188–189
 Decolonization, 312
 Decontamination agents, 585–586
 Definitive hosts, 667
 Dehydration, 873
 Delayed lactose fermenters, 185
Delftia acidovorans, 490
Delftia spp., 490
Delftia tsuruhatensis, 490
 Delta hepatitis virus, 745

- Deltavirus*, 745
 Denaturation, 232
 Dendrograms, 244, 245f
 Dengue, route of transmission, 33f
 Dengue fever (DF), 733, 858f
 Dengue hemorrhagic fever (DHF), 733
 Dengue virus, 709–710
 Density, of colony, 175, 175f
 Density measurement, 16
 Dental biofilms, 782–784
 Dental plaque, 782–783
 Deoxynucleotide triphosphates (dNTPs), 232
 Deoxyribonucleases (DNases), 190
 Deoxyribonucleic acid (DNA), 190
 anatomy of, 19–21, 20f
 antibiotic interference with replication of, 258
 antibiotic interference with transcription of, 258
 double-stranded, 718–727
 family Streptococcaceae and, 325
 single-stranded, 727–728
 Southern blot and, 230f
 template, 232f
 Department of Health and Human Services (DHHS), 755–756
Derma-centor variabilis, 553, 553f
 Dermatitis, 834–837
 Dermatomycoses, 602, 602t
 Dermatophytes, 604, 836
 Dermatophytic infections, 602
 Dermatophytoses, 602, 836, 837f
 Dermis, 833, 834f
 Descemet membrane, 1009
 Destruction, 66–67
 Detection limit, 116
 Detergents, 73
 DFA. *See* Direct fluorescent antibody
 Diabetes, 981
 Diabetic foot infections, 841–842, 842b
 Diagnostic laboratory tests, evaluation and interpretation of, in microbiology laboratory, 116
 Diagnostic sensitivity, 116–117
 Diagnostic specificity, 117
Diamanus montanus, 769
 Diapedesis, 41
 Diarrhea, 870
 acute, 872
 Aeromonas spp. and, 464
 bacterial pathogens causing, 875–879, 875b, 879b
 chronic, 872
 Clostridioides difficile and, 503, 878, 886
 common pathogens in, 872t
 Cryptosporidium parvum and
 Cryptosporidium hominis, 880
 culture for, 884–886
 Cyclospora cayetanensis and *Cystoisospora belli*, 880
 cytomegalovirus (CMV) and, 882
 diagnosis of, 871–873
 Entamoeba histolytica and, 880, 880f
 enterotoxin-mediated, 873–874, 873b, 874b
 Escherichia coli and, 422, 877, 877t, 886f
 in food poisoning, 367
 food vehicles and, 872t
 Giardia duodenalis, 879–880
 Helicobacter species and, 878, 879f
 history for, 871–873
 invasion of bowel mucosal surface and, 874, 874b
 invasion of full-bowel thickness with lymphatic spread and, 874b
 laboratory diagnosis of, 883–886
 laboratory studies for, 873, 873b, 873f
 Listeria monocytogenes and, 878
 media for, 885t
 Microsporidia and, 880, 880b
 opportunistic pathogens and, 881
 parasitic pathogens and, 879–881, 879b
 persistent, 872
 physical examination for, 873
 Salmonella species and, 876, 884, 884f
 Shigella species and, 876, 884f
 in special circumstances, 881–883
 traveler's, 872, 881, 883f
 treatment and prevention of, 886–888
 Vibrio species and, 877–878, 886, 886f
 viral pathogens causing, 875b
 Yersinia enterocolitica and, 878, 886
 Diarrheagenic strains, 32–34
 Dideoxynucleotide nucleotides (ddNTPs), 244
 Dienes stain, 564–565, 565f
Dientamoeba fragilis, 659t, 661, 661f
 Differential media, 14, 131
 Differential stains, 142–143, 143t
 Diffusely adherent *Escherichia coli* (DAEC), 423t, 877
 Digestion agents, 585–586
 Digital PCR, 237
 Dihydrofolate, 258
 Dihydrolase test, 188–189
 Dimorphic fungi, 4–6, 600
 Dimorphism, 600
 Diphtheria, 348–349
 laboratory diagnosis of, 350–351
 route of transmission, 33f
 treatment of, 351
 Diphtheria toxin, 349
 Diphtheroids, 174–175, 175f, 348
Diphyllobothrium latum, 688, 688t, 689f
 Diploid, 716
Dipylidium caninum, 690, 692f
 Direct antigen detection assays, 222–224, 222t
 Direct detection method, for viral infections, 714–716
 Direct examination
 of *Bacillus anthracis*, 761, 762f
 of bacterial infection, 157, 157f
 of *Burkholderia* spp., 763, 763f
 of contaminating materials, 155, 155f, 156f
 of *Francisella tularensis*, 766
 of gram-negative bacillary infections, 164, 164f, 165f, 166f
 of gram-negative bacterial infections, 162, 162f, 163f
 of gram-positive bacillary infections, 158, 158f, 159f
 with filaments and branches, 160, 160f, 161f
 uncommon, 159, 159f, 160f
 of *Yersinia pestis*, 770–771, 771f
 Direct fluorescent antibody (DFA) test, 210–211, 211f, 550–551, 645
 for *Legionella*, 413f
 for viral infections, 715
 Direct microscopic examination, 131
 of anaerobes, 507
 of Enterobacterales, 440
 of fungi, 628–630
 of *Neisseria gonorrhoeae*, 375, 376f
 of *Neisseria meningitidis*, 381, 382f
 of ocular infections, 1016–1017, 1017f, 1017t
 of *Chlamydia psittaci*, 549–551, 551f, 552f
 Direct plate count, 16
 Direct sandwich immunoassay, 212, 214f
 Direct tube coagulase, 918
 Direct-acting antiviral (DAA) therapy, 748
 Directly observed therapy (DOT), 823
 Disaggregation, biofilms and, 782, 782b
 Disinfectants, 66–67
 antiseptics *versus*, 71–75
 compatibility of, 68–69
 concentration of, 68
 Mycobacterium spp. and, 583
 Disinfection, 66
 biofilms and, 68
 compatibility of disinfectants in, 68–69
 concentration of disinfecting agent and, 68
 contact time and, 68
 degree of killing in, factors influencing, 67–69
 methods of, 69–71, 69t
 chemical, 70
 physical, 69–70
 nature of surface for, 68
 organic material and, presence of, 68
 pH and, 68
 sterilization *versus*, 66–67
 temperature and, 68
 Disk diffusion susceptibility testing, 490
 Disk diffusion testing, 279–282, 279f, 281f, 282f
 Disk storage, 280
 Dispersion B (DspB), 787
 Disseminated intravascular coagulation (DIC), 861–862, 863f, 909
 Disseminated strongyloidiasis, 698
 Dissemination, 37
 of resistance determinants, 267–268, 267b
 Distorted grin, 502
 DiversiLab System, 243
 Division septum, bacteria, 5f
 DNA. *See* Deoxyribonucleic acid
 DNA microarrays, 244–245
 DNA polymerase, 232
 DNA replication, 258
 DNA synthesis inhibitor, 253t–254t
 DNA transcription, 258
 Dog bites, 841
 Dog tapeworm, 692
Dolosicoccus spp., 338–339
Dolosigranulum spp., 339
 Donovan bodies, 962–963, 963f
 Donovanosis, 962–963, 962f, 963b
 Doripenem, 255–256
 Dot blots, 218–219
 Double immunodiffusion, 206

- Double-stranded deoxyribonucleic acid (dsDNA), 718–727
- Double-stranded ribonucleic acid (dsRNA), 728–729
- Doubling time, 15
- Doxycycline, 253t–254t
- Dracunculiasis, 860
- Dracunculus medinensis* (guinea worm), 703–706
- Droplet digital PCR assays, 237
- Dry heat, 70
- DrySpot Campylobacter test kit, 471
- Dual-probe fluorescence resonance energy transfer, 235
- Dual-probe FRET, 235f
- Duodenal aspirates, 643
- Duplex formation, 228
- Durham tube, 191
- Dysentery, *Shigella dysenteriae* causing, 437
- Dysgonic fermenters, 403
- Dysuria, 374, 927–928, 932
- D-zone test, 285, 289f, 322, 322f
- E**
- Ears, specimen collection from, 125t–126t
- East African sleeping sickness, 664
- Eastern equine encephalitis (EEE), 742–743, 894
- Eaton agent, 559
- Ebola virus, 732, 732f, 764–766, 765f
- Ebolavirus Sudan strain (EBO-S), 733
- Ebolavirus Tai Forest strain, 732
- Ebolavirus Zaire strain (EBO-Z), 733
- Ecchymoses, 835b
- Eccrine glands, 28
- ECF. *See* Extended care facility
- Echinococcosis (hydatid cyst disease), 692
- Echinococcus granulosus*, 692, 897
- Echoviruses, 739, 854
- Ecthyma gangrenosum, 847–848
- Ectoparasites, 861, 862f
- Ecuyer, Simon, 754–755
- Eczema, 855–856, 856f
- Edema factor, 364
- Education
- continuing, 106
 - infection control and, 62, 62b
 - laboratory scientists and infection prevention and control practitioners, 62
 - safety, 62
- Edwardsiella*, 432
- Edwardsiella tarda*, 432
- Efflux pumps, 262–264, 262f
- Egg yolk agar (EYA), 517
- Egg-based media, 587
- Ehrlich indole test, 491
- Ehrlichia* spp., 555, 555f
- Ehrlichiosis, 555, 996
- Ehrlich's reagent, 190, 190f
- EIA. *See* Enzyme immunoassays
- Eikenella corrodens*, 177, 402, 402f
- El Tor, 458
- Electrical safety, 97
- Electrophoresis
- agarose gel, 230, 233f
 - multilocus enzyme, 242
 - PCR and, 230
 - pulsed-field gel, 242, 242f
 - Western blots and, 218
- Elek test, 351, 353f
- Elementary body (EB), 544, 546f
- Elevation, colony morphology and, 175, 175f
- Elizabethkingia meningoseptica*, 490–491
- Embden-Meyerhof-Parnas (EMP) pathway, 17, 17f, 18b, 185, 185f
- Embryo, hexacanth, 687–688
- Emergency response plans, in infection control, 62–63
- Emerging pathogens, 51
- Emerging viral respiratory tract infections, 809–811
- Emerging zoonoses, 998–999, 998b
- Empedobacter* spp., 490
- Empiric antimicrobial therapy, 795
- Employee right-to-know, 85–86
- Empyema, 333, 806t–807t, 817–819
- case study on, 817b
 - causes of, 817
 - clinical manifestations of, 817–818
 - complications with, 818
 - epidemiology of, 817
 - laboratory diagnosis for, 818
 - pathogenesis of, 817
 - treatment for, 818
- Encephalitis, 894
- central nervous system infections and, 898–899
 - Eastern equine, 894
 - herpes simplex virus *versus*, 720
 - meningoencephalitis, 894
 - meningoencephalitis and, 898–899
 - St. Louis, 894
- Encephalitozoon intestinalis*, 681
- Endemic relapsing fever, 536–537
- Endemic syphilis, 538, 541
- Endocarditis
- Aggregatibacter aphrophilus* and, 399
 - Cardiobacterium hominis* and, 401
 - infective, bacteremia and, 910
 - Lactobacillus* spp. and, 504
 - Pseudomonas mendocina* and, 484
 - Stenotrophomonas maltophilia* and, 485–486
- Endogenous anaerobes, 498, 499t
- Endogenous microbiota, 498
- Endogenous opportunistic pathogens, 975
- Endolimax nana*, 650t, 652, 653f
- Endometritis, 546–547
- Endophthalmitis, 1002, 1014, 1014b
- Endospore stain, 13
- Endospores, 8, 501, 502f
- Endotoxins, 9
- pathogenesis and, 37, 39, 39t
- Endotracheal tube, 53–54
- Engineering controls, 79
- Enriched broth, 14
- Enriched media, 14, 131
- Enrichment broth, 131
- Entamoeba coli*, 650t, 652, 652f
- Entamoeba hartmanni*, 650t, 651, 651f
- Entamoeba histolytica*, 26
- clinical infections of, 648–649
 - laboratory diagnosis of, 649–651, 649f, 650t, 651f
 - related organisms and, 647–651
- Entamoeba moshkovskii*, 647–648
- Enteric adenoviruses, 719, 875
- Enteric fevers, 876
- Salmonella* causing, 434–435
- Enterics, 419
- Enteritis necroticans, 502
- Enteroadherent *Escherichia coli* (EAEC), 423t
- Enteroggregative *Escherichia coli*, 877, 877t
- Enterobacter*, 427
- Enterobacter cloacae*, 427, 428t
- Enterobacterales, 417, 418b, 452b
- antimicrobial resistance in, 419–421
 - classification of, 418–419, 419t, 420t
 - clinical significance of, 419, 420t
 - colony morphology of, 418
 - general characteristics of, 418–421, 418b
 - identification of, 441–442, 443f, 445t–451t
 - isolation of, 440
 - laboratory diagnosis of, 439–452
 - microscopic morphology of, 418
 - opportunistic members of, 421–432
 - other genera of, 439
 - primary intestinal pathogens of, 432–439
 - screening stool cultures for pathogens of, 440–442, 441t
 - serologic grouping in, 442–452
 - specimen collection and transport of, 439
 - virulence and antigenic factors of, 419
- Enterobius vermicularis* (pinworm), 693–694, 693t, 694f
- Enterococcus avium*, phenotype and biochemical characteristics of, 337t
- Enterococcus casseliflavus*, phenotype and biochemical characteristics of, 337t
- Enterococcus durans*, phenotype and biochemical characteristics of, 337t
- Enterococcus faecalis*, 337–338
- classification of, 327t
 - phenotype and biochemical characteristics of, 337t
- Enterococcus faecium*
- classification of, 327t
 - phenotype and biochemical characteristics of, 337t
- Enterococcus gallinarum*, phenotype and biochemical characteristics of, 337t
- Enterococcus raffinosus*, phenotype and biochemical characteristics of, 337t
- Enterococcus* spp., 336–338, 336f, 337f
- antimicrobial resistance and, 338
 - biochemical identification of, 340–344, 341f
 - high-level aminoglycoside resistance in, 290
 - susceptibility testing and, 289–290, 289f
- Enterocytozoon bienersi*, 681
- Enterocytozoon intestinalis*, 681
- Enterohemorrhagic *Escherichia coli* (EHEC), 424, 877, 877t
- Enteroinvasive *Escherichia coli* (EIEC), 423t, 424, 877, 877t
- Enteropathogenic *Escherichia coli* (EPEC), 423t, 424, 877, 877t
- Enterotoxigenic *Escherichia coli* (ETEC), 422–423, 423t, 877, 877t
- Enterotoxin-mediated diarrhea, 873–874, 873b, 874b
- Enterotoxins
- Bacillus cereus* and, 368t
 - Staphylococcus aureus* and, 310–311
 - from *Vibrio cholerae*, 32–34

- Enteroviral infections, 854
 Enteroviruses, 738–739, 739b, 854, 894
 Entner-Doudoroff pathway, 17, 18b, 18f, 185, 185f
 Entomophthoromycosis, 843t, 845
 Enveloped virus, 710
 Environmental cultures, outbreak investigations and, 60–61, 60b
 of air, 60
 of surfaces, 61
 of water, 60–61
 Environmental Protection Agency (EPA), chemical surface disinfectants and, regulations on, 70, 75, 75b
 Enzymatic inactivation
 of antimicrobial agents, 265–266
 resistance and, 262–263, 263f
 Enzymatic target site alteration, 264–265
 Enzyme immunoassays (EIAs), 212–215, 214f, 303, 522, 551
 parasites and, 645
 in viral infections, 715, 715f
 Enzyme-linked immunosorbent assay (ELISA), 213, 215f, 728, 955
 for *Escherichia coli*, 425
 Enzyme-linked virus-inducible system (ELVIS), 721
 Enzymes, 517, 520
 ability to produce, 37–39
 EPA. *See* Environmental Protection Agency
 Epidemic, defined, 56–57
 Epidemic keratoconjunctivitis (EKC), 1007
 Epidemic relapsing fever, 536–537
 Epidemiologic curve, 57, 57f
 Epidemiologic cutoff values (ECVs), 273, 636
 Epidermis, 833, 834f
 Epidermolytic toxin, 311
Epidermophyton floccosum, 605
 Epididymitis, 946–947, 971
 Epiglottitis, 795, 797t, 802–804, 804b
 case study on, 802b
 causes of, 803
 clinical manifestations of, 803
 complications of, 803
 epidemiology of, 802–803
 from *Haemophilus influenzae*, 393
 laboratory diagnosis of, 803, 803f
 pathogenesis of, 803
 treatment for, 804
 Epimastigotes, 665
 Epithet, 6
 Epitopes, 201
 Epstein-Barr virus (EBV), 722–724, 723f, 979
 direct hemagglutination test for, 209
 serologic test for, 723–724, 723t
 skin infections and, 856
Eptesicus fuscus, 731
 Equipment maintenance, in quality control, 110–111, 110t
 Erwiniaceae, 430
 Erysipelas, 328, 838–839, 838t, 839f
 Erysipeloid, 34t, 357, 839, 986–987, 986b, 987f
Erysipelothrix rhusiopathiae, 356–358, 358f, 359t, 986, 987f
 Erythema migrans (EM), 537, 848, 848f, 993
 Erythrasma, 836
 Erythrocytic phase, 670
 Erythromycin, 253t–254t, 322
 Erythromycin ribosome methylase (ERM), 264
 Eschar, 365, 760, 988
Escherichia albertii, 425–426
Escherichia coli, 32–34, 36t, 38t, 421–426, 421f
 causing extraintestinal infections, 422
 diarrhea and, 877, 877t, 886f
 disk diffusion testing for, 281f
 enteroaggregative, 423t
 enteroaggregative, 877, 877t
 enterohemorrhagic, 877, 877t
 enteroinvasive, 423t, 424, 877, 877t
 enteropathogenic, 423t, 424, 877, 877t
 enterotoxigenic, 422–423, 423t, 877, 877t
 gastrointestinal, 422–425, 423t
 Shiga toxin-producing, 424–425
 uropathogenic, 422, 423t
Escherichia fergusonii, 425–426
Escherichia/Citrobacter-like organisms, 172b, 173f
 Esculin hydrolysis, 489–490
 Esophagus, normal microbiota of, 29
 Etest, 286–287, 292, 292f, 528
 Ethidium bromide, 233f
 Ethylene oxide, 74
 Etiologic agent, 126
 Eugonic oxidizer, 490
 Eukarya, 6–7
 Eukaryotes
 cell structure of, 10
 cell envelope, 11
 cytoplasmic, 10–11
 classification by, 7
 prokaryotes and, 4, 4t–5t, 5f
 Eumycotic mycetomas, 361, 602, 607–608, 608t
 Eustachian tube, 801
Ewingella, 439
 Exanthems, 854
 Exfoliative toxin, 311
 Exoerythrocytic phase, 669–670
 Exogenous anaerobes, 498
 Exogenous opportunistic pathogens, 975
Exophiala dermatitidis, 609, 610f
Exophiala spp., 609, 609f
 Exopolysaccharide (EPS), 779
 Exotoxins, pathogenesis and, 37, 38t, 39, 39t
 Expecterated sputum, 126
 Exposure control plan, 78
 Extended care facility (ECF)
 frequently identified microbes in, 56, 56t
 infection control in, 51, 52t, 56
 Extended spectrum β -lactamases (ESBLs), 290–291, 291f, 816
 Extensively drug-resistant tuberculosis (XDR-TB), 575
 External membrane surface, plasma membrane, 8f
 Extrachromosomal DNA elements, 21
 Extrapulmonary tuberculosis, 573
 Extremophiles, 7
 Eye infections, *Bacillus cereus* causing, 368
 Eyelid infections, 1008–1009
 Eyes, specimen collection from, 125t–126t
- F**
 Facilitator, 109
Facklamia spp., 339
 Facultative anaerobes, 15, 350, 497, 497t
 False-negative test, 203–205, 204f
 False-positive test, 200, 204–205, 204f
 Family, 6
 Farcy, 762–763
Fasciola hepatica, 683, 684t
Fasciolopsis buski, 682–683, 684t
Fasciolopsis buski/Fasciola hepatica eggs, 683, 684f
 Fast-acting antiseptic, 76–77
 Fastidious bacteria
 infrequently encountered or, 287
 modified methods for, 284–287
 Fastidious organisms, 170
 FDA. *See* Food and Drug Administration
 Fecal leukocytes, 873
 Fecal microbiota transplant (FMT), 529–530
 Fecal specimens, 640–643
 collection, handling, and transport of, 640–641
 macroscopic examination of, 641–642, 642f
 microscopic examination of, 642–643
 mycotic sample collection of, 628
 preservation of, 641, 641t
 specimen collection from, 125t–126t
 Fermentation, 16–17, 185
 carbohydrates utilization and, 185
 Fermentative gram-negative bacilli, 475
 Fetus, immunocompromised patients and, 981–982
 Fever, 729
 blackwater, 668
 Fiery serpent, 703
 Fifth disease, 728
 Filamentous form, of colony morphology, 174–175, 174f
 Filamentous hemagglutinin (FHA), 412
 Filarial worms, 701–703
 Filariasis, 859–860, 860f
 Filariform larvae, 644, 693t
 Filoviridae, 732–733, 764–765
 Filtration, disinfection and sterilization with, 70
 Fimbriae, 10, 35, 326f
 Finite cell cultures, 716
 Fire safety, 96–97, 97t
 First aid training, 97
 First-generation screening tests, 740–741
 Fitz-Hugh-Curtis syndrome, 374
 Flagella, 5f, 10, 10f
 lophotrichous, 10
 monotrichous, 10
 periplasmic, 534
 peritrichous, 10
 polar, 10
 Flagellates
 blood, 662–667, 663f
 nonpathogenic intestinal, 662
 pathogenic intestinal and urogenital, 658–662, 658f, 659t
 Flat warts, 968
 Flaviviridae, 733–734
 Flavobacteriaceae, 490–491
 Flea vectors, 754, 755f

- Fleas, 553, 992, 992f
 Flocculation tests, 206
 Flocked swabs, 735–736
 Flow cell, 787
 Flukes (trematodes), 682–687, 683f, 684t
 blood, 685–687
 clinical infections of, 685–686
 laboratory diagnosis of, 686–687, 687f
 life cycle of, 686, 686f
 intestinal, 682–683, 683f
 lung, 685
 Fluorescein isothiocyanate (FITC), 210
 Fluorescence, 514–515, 515f, 515t, 516f
 Fluorescence in situ hybridization, 918, 918f
 Fluorescence resonance energy transfer (FRET), 233–234
 dual-probe, 235f
 Fluorescent antibody, direct, 645
 Fluorescent immunoassay, 303
 Fluorescent polarization immunoassay, 303
 Fluorescent treponemal antibody
 absorption (FTA-Abs) test, 212
 Fluorochrome, 205
 Fluorochrome stain, 13, 210
 Fluorogenic substrates, 193–194
 Fluorophore, 233
 Fluoroquinolones, 253t–254t
 Focused monitors, 107
 Folate synthesis, inhibition of, 258, 258f
 Folliculitis, 313, 838t, 840
 Fomites, 32
Fonsecaea compactum, 607t
Fonsecaea pedrosoi, 607t
 Food, transmission by, 32–34
 Food and Drug Administration (FDA),
 237–238, 274, 490–491, 551, 636, 948t
 chemical skin antiseptics and, regulation
 on, 70, 75–77
 Food poisoning, 869, 869b–870b, 870b, 887b,
 887b–888b
 Bacillus cereus causing, 367
 Clostridium perfringens and, 501–502
 Salmonella species and, 876
 Staphylococcus aureus and, 313–314
 toxic agents of, 881, 881b, 882t
 Vibrio parahaemolyticus and, 459
 Foot infections, diabetic, 841–842, 842b
 Foreign bodies, opportunistic
 microorganisms and, 31t
 Forensic microbiology, 754b, 772–774, 773b
 Formaldehyde, 72
 Formalin, for fecal samples, 641t
 Frame, 22
Francisella, 406–407
Francisella tularensis, 407, 407f, 766–767, 767f,
 989b
 from blood culture, 916
 isolation of, 135t
 Franklin, Rosalind, 19
 Free ribosome, 5f
 Fuller, John, 729
 Fulminant meningococemia, 381
 Fungal infections
 central nervous system and, 895–896,
 896b, 896f, 902
 Fungal scrapings, specimen collection from,
 125t–126t
 Fungemia, 908, 917
 Fungi, 4–6
 antifungal susceptibility testing and,
 635–636
 cancer patients and, 977
 case on, 598b
 clinically significant species of, 603–626
 conjunctivitis and, 1008
 dimorphic, 4–6, 600
 endophthalmitis and, 1014
 general characteristics for, 598–600
 identification of, 631–635
 immunodiagnosis of, 635
 isolation methods of, 630–631
 keratitis and, 1010–1011, 1011b
 laboratory diagnosis of, 626–635, 627f,
 627t
 medically significant, 597
 pneumonia and, 821–822
 polymorphic, 600
 reproduction of, 600, 600f
 skin infections from, 844–845, 852–853
 systemic dimorphic, 852–853, 852f
 taxonomy of, 600–601
 UTI and, 930–931, 931b, 935
 Furuncles, 313, 838t, 840, 841f
Fusarium falciforme, 608
Fusarium spp., 619, 619b, 619f, 853, 1017
 Fusiform, 11
Fusobacterium mortiferum, 526, 526f
Fusobacterium nucleatum, 526
Fusobacterium spp., 504, 526
G
 Galactomannan, 634
 β -Galactosidase, 185
 β -Galactoside permease, 185
 Gametes, 668
 Gametocytes, 644
 Gardasil, 726, 968
Gardnerella vaginalis, 358–360, 360t
 Gas gangrene, 501, 842, 842f
 Gases, 74–75
 Gas-liquid chromatography (GLC), 520
 GasPak EZ Gas Generating Container
 System, 469
 GasPak EZ Gas Generating Pouch System,
 469
 GasPak jar, 511
 testing frequency of, 110t
 GasPak Pouches, 506
 Gastric aspirates, 584
 Gastric pH, 870
 Gastritis, type B, 468
 Gastroenteritis, 728
 Giardia duodenalis and, 658
 Salmonella spp. and, 434, 876
 Vibrio cholerae and, 458
 Gastrointestinal anthrax, 365, 760–761,
 988–989
 Gastrointestinal histoplasmosis, 883
 Gastrointestinal infections, 869, 869b–870b,
 870b, 887b, 887b–888b. *See also*
 Diarrhea; Food poisoning
 anatomic considerations for, 870–871,
 871f
 diagnosis of, 871–873
 host defense for, 871
 laboratory diagnosis of, 883–886
 Gastrointestinal tract
 anaerobes and, 500
 anatomy of, 870, 871f
 cleansing mechanism of, 40
 Escherichia coli in, 422–425, 423t
 normal microbiota of, 29, 30b
 GB virus type C (GBV-C), 748
 GBS. *See* Group B streptococci
 GC-LECT medium, 376t
 Gelatin hydrolysis, 483
Gemella spp., 339
 Gemifloxacin, 253t–254t
 Gene transfer, 782
 Gene transfer, mechanism of, 22–24
 General laboratory safety, 78–98, 78b
 Generally recognized as safe and effective
 (GRASE), 72
 Generation time, 15
 Genes, 10
 Genetic elements, alteration and, 21–22
 Genetic recombination, 22
 GeneXpert system, 238
 Genital herpes, 720, 954b
 clinical manifestations of, 954–955, 955f
 epidemiology of, 954
 laboratory diagnosis of, 955–956, 955f
 overview of, 954–956
 treatment of, 956
 Genital infections and sexually transmitted
 infections, 942, 943b
 acquired immunodeficiency syndrome,
 963–967
 cervicitis, 948–949
 epididymitis, 971
 genital ulcer disease, 953–963
 genital warts, 968–969
 molluscum contagiosum, 971
 pelvic inflammatory disease, 970–971
 proctitis, 971
 urethritis, 945–948
 viral hepatitis, 969–970, 970f
 vulvovaginitis, 949–953
 Genital ulcer disease (GUD), 394, 953–963.
 See also Syphilis
 Genital warts, 968–969, 968f, 969b
 Genitalia, specimen collection from, 125t–126t
 Genitourinary tract
 aerobes and, 500
 cleansing mechanism of, 40
 normal microbiota of, 29–30, 30b
 Genitourinary tract infections, associated
 with mollicutes, 561–562, 562b
 Genotype, 6
 classification by, 7
 GenoType Mycobacteria Direct test, 594
 Gen-Probe, 948t
 Gentamicin, 253t–254t, 277t
 Genus, 6
 Geophilic fungi, 605
Geotrichum spp., 619, 619f
 Geriatric population, UTI in, 925–926, 926b,
 926f
 Germ theory of disease, 71
 Germ tube production, 632, 633f
 Giant intestinal fluke, 682–683
Giardia duodenalis, 658–661, 659t, 879–880
 clinical infection of, 658–660
 laboratory diagnosis of, 660–661, 660f

- Giardia intestinalis*, 658
Giardia lamblia, 658
 Giardiasis, direct antigen detection assay for, 223–224
 Giemsa stain, 630
 Gilchrist disease, 612
 Gingivitis, 782
 Glanders, 487, 762–763
Globicatella spp., 338–339
 Glomerulonephritis, acute, 329, 798
 Glove boxes, 510, 510f
 (1,3)- β -D-glucan test, 634
 Glucocorticoids, for bacteremia, 920
 Glucose, 184, 185f
 metabolism, 187–188
 β -d-glucuronidase, 343
 Glutamate dehydrogenase (GDH), 522
 Glutaraldehyde, 72
 Glycocalyx, 10
 Glycolipid, plasma membrane, 8f
 Glycolipopeptides, 253t–254t
 Glycolysis, 185, 185f
 Glycopeptides, 253t–254t, 256
 Glycoprotein, plasma membrane, 8f
 Glycylcyclines, 259
Gnathostoma spinigerum, 897
 Golgi apparatus, 5f
 Gomori methenamine silver (GMS) stain, 822, 822f
 Gonococcal Isolate Surveillance Project (GISP), 380
 Gonococcal urethritis, 945
 Gono-Pak, 375
 Gonorrhea, 374, 943–944
 clinical infections of, 374–375, 375b, 945
 epidemiology of, 374, 945
 FDA-approved molecular tests for, 948t
 laboratory diagnosis of, 375–380
 reported cases of, 945, 946f
 treatment of, 380, 948
Gordonia, 363, 363t
 Grading, microscopic examinations and material, 149–151
 Graft-versus-host disease (GVHD), 826
 Gram, Christian, 140
 Gram-negative archaea, 7
 Gram-negative bacillary infections, direct examination of, 164, 164f, 165f, 166f
 Gram-negative bacteria, 140
 Gram-negative bacterial infections, direct examination of, 162, 162f, 163f
 Gram-positive archaea, 7
 Gram-positive bacillary infections, direct examination of, 158, 158f, 159f
 with filaments and branches, 160, 160f, 161f
 uncommon, 159, 159f, 160f
 Gram-positive bacteria, 140
 Gram smear report, 150
 Gram staining, 11–13, 12f, 13b, 140, 140f
 for *Legionella*, 409
 for *Mycobacterium* spp., 586, 587t
 Gram-negative bacilli, 929
 Gram-negative bacteria, 252, 255f
 biochemical identification of, 183
 amino acid utilization, 188–189, 189f
 carbohydrate utilization, 184–187, 185f, 186t, 194, 194t, 195f
 Gram-negative bacteria (Continued)
 glucose metabolism and, 187–188
 KIA and, 186, 186t, 187f
 manual multitest systems for, 192–193, 192t
 miscellaneous tests for, 189–192, 190f
 O/F tests and, 185–186, 185f, 186t
 ONPG tests and, 187, 187f
 rapid and automated identification systems for, 193–197, 194t, 195t
 TSI and, 186–187, 186t, 187f
 efflux pumps and, 262, 262f
 identification of anaerobic, 518, 519f, 519t, 520f, 524–526, 525t
 Gram-positive bacilli
 aerobic, 347, 348b, 349f
 pleomorphic, 350
 UTI and, 930
 Gram-positive bacteria, 252, 255f
 differentiation of, 317t
 identification of anaerobic, 517–518, 518b, 518f, 518t, 523–524, 523t
 Gram-positive cocci
 Streptococcus, *Enterococcus*, and other
 catalase-negative, 324–325, 324b–325b, 345–346, 345b–346b
 biochemical identification of, 340–344, 341f
 laboratory diagnosis of, 340–345
 susceptibility testing of, 345
 UTI and, 929–930
 Gram-stained smear, 172b
Granulicatella spp., 338, 916
 Granulocytes, 825–826
 Granulocytopenia, 977
 Granuloma, 572
 Granuloma inguinale, 962, 963b, 963f
 GRASE. *See* Generally recognized as safe and effective
 Gravid proglottids, 687, 687f
 Gray patch ringworm, 605
 Grocott-Gomori methylene silver (GMS) stain, 599–600
 “Ground-glass” appearance, 409–410
 Group A streptococci (GAS) pharyngitis, 222, 796, 798
 Group A streptococcus, testing of throat samples for, 118
 Group B streptococci (GBS)
 penicillin and, 330–332
 Streptococcus agalactiae and, 330
 Group C streptococci, 327t, 332–333
 Group G streptococci, 332–333
 Growth, 13–16
 bacterial, 15–16
 environmental factors influencing, 15
 nutritional requirements for, 14–15
 Growth curve, 15–16, 16f
 Growth media, types of, 14–15
 Growth rate, *Mycobacterium* spp. and, 589–590
 Guanosine-triphosphate (GTP), 18
 Guillain-Barré syndrome (GBS),
 Campylobacter species and, 468
 Guinea worm (fiery serpent), 703
 Gummas, 539, 958, 958f
- ## H
- H antigen, 419, 433
 H1N1 influenza A, 710, 735, 809
 H3N2 influenza, 710, 735
 H5N1 influenza (avian flu), 735
 H7N9 influenza, 735
 HACEK group, 399–403, 917
 key reactions and characteristics of, 400t
Haemophilus, 391–392
 biochemical tests for, 397, 398t, 399t
 colony morphology of, 395
 general characteristics of, 391–392
 laboratory diagnosis of, 394–397
 laboratory identification of, 395–397
 microscopic morphology of, 395, 396f
 specimen processing and isolation of, 394–395
Haemophilus aegyptius, 394
Haemophilus ducreyi, 394, 394f
 isolation of, 135t
 Haemophilus ID Quad Plate, 396
Haemophilus influenzae, 36t, 391b, 392–399
 adherence mechanisms of, 393
 bacterial tracheitis from, 393
 capsule and, 392
 differential tests for, 399t
 epiglottitis from, 393
 historic perspective on, 392–394
 immunoglobulin A proteases in, 392
 infections
 clinical manifestations of, 393–394, 393b, 393t
 treatment of, 397
 meningitis from, 393
 nontypable, 392
 virulence factors of, 392–393
Haemophilus influenzae biogroup *aegyptius*, 394
Haemophilus influenzae type B (Hib), 802–803
Haemophilus parainfluenzae, 394, 399t
Haemophilus spp., 171–172
Haemophilus test medium (HTM), 285
Hafnia, 431
 Hafniaceae, 431–432
 HAI. *See* Healthcare-associated infection
 Hair, 626
 Hair perforation test, 632, 632f
 Halogens, 72
 Halophiles, 7
 HA-MRSA. *See* Hospital-associated methicillin-resistant *Staphylococcus aureus*
 Hand, foot, and mouth disease (HFMD), 739
 Hand hygiene, 62, 840
 Handwashing, hygienic, 76–77, 76b
 Hansen disease (leprosy), 571, 580, 852, 852f
Hantavirus, 729–730
 Hantavirus pulmonary syndrome, 730, 730t
 Hazardous chemicals, 95b
 classification of, 95–96
 laboratory safety for, 96
 Hazardous waste, reduction of, 85, 85f, 86f
 Hazard-rating diamond, 85–86
 Health care antiseptic drug products, 72
 Health care personnel handwash, 75, 76t
 Healthcare-associated infection (HAI), 51, 795, 976

- Heat, disinfection and sterilization with,
69–70
dry, 70
moist, 69–70
- Heat resistance factors, 39–47
physical barriers as, 39
- Heavy metals, 74
- Heineman's method, 149
- Hektoen enteric (HE) agar, 171*t*, 418, 440, 441*f*, 884*f*
- Helicobacter pylori*, 878, 879*f*
clinical manifestation of, 468
isolation of, 135*t*
- Helicobacter* spp., 470*t*
- Helminths, 682
central nervous system infections and, 897–898
skin infections from, 859–860
- Hemadsorption test, 717–718
- Hemagglutination, 209
- Hemagglutination inhibition, 209
- Hemagglutinin (H), 717–718
- Hemagglutinin-neuraminidase (HN)
antigen, 736
- Hematogenous pyelonephritis, route of
transmission, 33*f*
- Hematoproliferative disorders,
opportunistic microorganisms and,
31*t*
- Hemoglobin, 577
- Hemolysin, 460
- α -Hemolysin, 311
- β -Hemolysin, 311
- Hemolysis
colony morphology and, 173–174, 174*f*
in *Listeria monocytogenes* and, 356, 356*f*
Streptococcus spp., *Enterococcus* spp. and,
326, 326*b*, 326*t*
- α -Hemolysis, 173, 326, 326*f*, 326*t*
- β -Hemolysis, 173–174, 174*f*, 326, 326*f*, 326*t*
- α -Hemolytic viridans streptococci, 179*f*
- Hemolytic-uremic syndrome (HUS),
877–878
- Hemorrhagic fever, 764–765, 765*f*
- Hemorrhagic fever viruses, 858, 858*f*
- Hemorrhagic fever with renal syndrome
(HFRS), 729–730
- HEp2, 716–717
- Hepacivirus, 747–748
- Hepadnaviridae, 744–745
- Hepatitis, 220–221
viral, 969–970, 969*f*, 970*f*
- Hepatitis A virus (HAV), 743–744, 743*t*, 744*f*
- Hepatitis B virus (HBV), 715, 743–745, 743*t*,
745*b*, 746*f*, 746*t*
as bloodborne pathogen, 745
route of transmission, 33*f*
- Hepatitis C virus (HCV), 716, 743*t*, 747–748,
748*f*
- Hepatitis D virus (HDV), 743–747, 743*t*,
747*f*, 747*t*
- Hepatitis E virus (HEV), 748
- Hepatitis G virus (HGV), 743–744, 748
- Hepatitis viruses, 743–749
- Hepatovirus, 744
- Hepeviridae, 748
- Hepevirus, 748
- Heraclitus, 780
- Herd immunity, 749–750
- Herpes simplex virus (HSV), 715, 720, 827,
855–856, 856*f*, 945
CPE and, 717–718, 717*f*
cytopathic effect (CPE) and, 955
diagnosis of, 720–721, 721*f*
encephalitis *versus*, 720
types of, 720
- Herpes zoster, 724
- HerpeSelect 1 and 2 Immunoblot IgG, 955
- HerpeSelect HSV-1, 955
- Herpesviridae, 719–726, 854–856
human herpesvirus 6, 725
human herpesvirus 7, 725
human herpesvirus 8, 725–726
meningitis and, 895
- Herpetic whitlow, 855–856, 856*f*
- Heterolactic fermentation, 17
- Heterophile antibodies, 204, 212
- Heterophyes heterophyes*, 683, 684*t*
- Heteroploid, 716–717
- Heteroresistant strains, 296
- Heterotrophs, 14
- Hexacanth embryo, 687–688
- Hexachlorophene, 73–74
- Hidradenitis suppurativa, 840–841
- High-efficiency particulate air (HEPA),
827
- High-frequency recombination strains, 23
- High-level aminoglycoside resistance, 290
- Highly active antiretroviral therapy
(HAART), 741, 823–824, 883
- Highly pathogenic avian influenza (HPAI)
virus, 735
- High-pressure liquid chromatography
(HPLC), 580–583
- Hippurate hydrolase, 341
- Hippurate hydrolysis, of streptococci, 330,
331*t*, 341
- Histones, 10
- Histoplasma* spp., 600, 613–615, 614*f*
- Histoplasmosis, 613–614, 821–822
tuberculosis and, 820
- Hodgkin disease (HD), 723, 977–978
- Home care setting
frequently identified microbes in, 56
infection control in, 52*t*
- Homogenization, 132
- Homolactic fermentation, 17
- Hong Kong flu, 735
- Hookworms, 693*t*, 695–697, 697*b*, 697*f*, 698*f*,
860
- Horseradish peroxidase, 212
- Hortaea werneckii*, 604, 604*f*
- Hospital-acquired bacteremia, 908*t*
- Hospital-acquired pneumonia, 806*t*–807*t*,
815–817
case study on, 815*b*
causes of, 815
clinical manifestations of, 815–816
complications with, 816
epidemiology of, 815
laboratory diagnosis for, 816
pathogenesis of, 815, 816*f*
treatment for, 816–817
- Hospital-associated methicillin-resistant
Staphylococcus aureus (HA-MRSA),
320
- Host-parasite interaction, 25, 26*b*, 27*b*, 30*b*,
39*b*, 48*b*, 48*b*–49*b*
origin of microbial biota and, 26–27
- Host resistance factors
antimicrobial substances as, 40–41
cleansing mechanism as, 39–40
immune responses as, 42–47
inflammation as, 42, 43*f*
microbe mechanisms for overcoming,
47–49
phagocytosis as, 41–42
- Housekeeping genes, 243
- HSV-2 ELISA, 955
- Human blood bilayer Tween (HBT) agar,
360
- Human bocavirus (HBoV), 728
- Human granulocytic anaplasmosis (HGA),
556, 996
- Human herpesvirus 6 (HHV-6), 725, 856
- Human herpesvirus 7 (HHV-7), 725
- Human herpesvirus 8 (HHV-8), 725
- Human immunodeficiency virus (HIV),
709, 739, 740*b*, 740*f*, 742*b*, 823–825,
823*b*, 824*b*, 943. *See also* Acquired
immunodeficiency syndrome
acute infection of, 964
clinical manifestations of, 824
direct antigen detection assay for, 221,
224*b*
epidemiology of, 823–824
immunoblot, 741*f*
immunocompromised patients and,
975–976
laboratory diagnosis of, 824–825, 825*f*,
966–967
meningitis and, 895
ocular infections and, 1014
progression to AIDS, 965–966, 966*f*
respiratory pathogens and CD4 cell count
with, 823, 824*t*
serologic testing for, 221, 221*b*
treatment for, 825, 967, 967*t*
- Human metapneumovirus (HMPV), 728,
738
- Human monocytic ehrlichiosis (HME), 555,
996
- Human papillomavirus (HPV), 726, 726*t*,
857
- Human T-lymphotropic viruses, 739
- Human-to-human transmission, of
monkeypox virus, 727
- Humoral immune response, 45, 977
- Hyaline, phaeoid *versus*, 599–600, 599*f*
- Hyaluronidase, 328
- Hybrid capture, 232, 240
- Hybridoma, 203, 203*f*
- Hydatid cyst disease, 692
- Hydrogen peroxide (H₂O₂), 74
- Hydrops fetalis, 728
- Hydroxyl radical, 498
- Hymenolepis diminuta*, 689–690, 691*f*
- Hymenolepis nana*, 689–690, 691*f*
- Hymenolepis* spp., 689–690, 691*f*
- Hyperbaric oxygen chamber, 529
- Hyperinfection, 698
- Hyphae, 4–6, 598
- Hypotension, 905*t*, 910–911
- Hypothermia, 910–911

I

- Iatrogenic infections, 31, 52
- IgM antibody capture ELISA, 214, 215f
- IMDs. *See* Indwelling medical devices
- Immune complex, 201
- Immune responses, 42–47, 44t
 - adaptive, 975
 - cell-mediated, 46–47
 - cellular, 977
 - humoral, 45, 977
 - innate, 975
 - nature of, to infectious agents, 45
- Immune status testing, 205
- Immune system, 42–43
- Immune-mediated cutaneous disease, 861–863
- Immunity, 42–43
 - adaptive, 43–45
 - innate, 43, 45f
- Immunizations, 97
- Immunoassays, 303
 - for *Chlamydia psittaci*, 551
 - labeled, 210–219
 - of *Streptococcus* spp., 344
- Immunochromatographic tests (ICTs), 216
- Immunocompetent host, 974–975
- Immunocompromised patients
 - aging and, 981
 - AIDS and, 979
 - burns and, 979
 - cancer and, 977–978
 - CF and, 980–981
 - complement deficiency and, 979
 - definition of, 975
 - diabetes and, 981
 - diarrhea in, 881–883
 - HAI and, 976
 - HIV and, 975–976
 - infections in, 224
 - malignancy and, 977–979
 - opportunistic pathogens and, 974–975, 975t
 - organ transplantation and, 979–980
 - organisms associated with, 976t
 - postsplenectomy and, 980, 980b
 - pregnancy, fetus, neonates and, 981–982
 - respiratory tract infections in, 820
 - VAP and, 976–977
- Immunocompromised states, respiratory tract infections and, 825–827, 825b, 826b
- Immunofluorescent assays, 210–212, 211f, 212f
- Immunogenic substances, 201
- Immunogens, 43–44
- Immunoglobulin A (IgA), 46
- Immunoglobulin A proteases, 392
- Immunoglobulin D (IgD), 46
- Immunoglobulin E (IgE), 46
- Immunoglobulin G (IgG), 45–46, 46f, 46t, 201–202
 - immune responses and, 201–202, 202f
- Immunoglobulin M (IgM), 45–46, 46f, 46t, 201–202, 202f, 215f
- Immunoglobulins, 45
- Immunologic assays
 - for *Campylobacter* species, 471
 - for *Neisseria gonorrhoeae*, 379
- Immunosenescence, 981
- Immunosuppressed patients, listeriosis in, 355, 355b
- Immunosuppression, 974–975
 - opportunistic microorganisms and, 31t
- IMOVAX, 742
- Impermeability, resistance and, 261–262
- Impetigo, 313, 328, 837–838, 838f, 838t
- Impetigo contagiosa, 313
- Implant soak solutions, 136
- In situ amplification (ISA), 231
- In situ hybridization (ISH), 231
- Incidence, of disease, 117
- Inclusion body, bacteria, 5f
- Incubation, 280, 281f
 - conditions for, bacteremia and, 914
 - fungi and, 630
 - of specimen, 132–134
- Incubator, testing frequency of, 110t
- Index case, 57
- India ink stain, 12f, 13, 629–630
- Indifference, 303f
- Indigenous microbial biota
 - characteristics of, 26–27
 - skin and, 27–28
- Indirect agglutination, 206–207
- Indirect fluorescent antibody (IFA), 411
 - test, 210–211, 211f
- Indirect sandwich immunoassay, 212, 214f
- Individualized quality control plan (IQCP), 115–116
- Indole test, 190, 190f
- Induced resistance, 261
- Induced sputum, 126
- Indwelling catheter, specimen collection from, 125t–126t
- Indwelling medical devices (IMDs), 781, 785t
- Infant botulism, 764, 764f
- Infection control
 - education and, 62, 62b
 - laboratory scientists and infection prevention and control practitioners, 62
 - safety, 62
 - frequently identified microbes in, 55–56, 56t
 - acute care setting, 56
 - ambulatory care setting, 56
 - communal living, 56
 - extended care facility and home care setting, 56
 - public health and community setting, 55–56
 - general concepts in, 51–56
 - health care settings in, 52, 52t
 - laboratory role in, 50, 51b, 62b–63b, 63b
 - outbreak investigation in, 56–62, 57b, 57f
 - antibiograms and, 59, 59t
 - cultures and serology, 59–60
 - environmental cultures and, 60–61, 60b
 - laboratory support for, 59
 - local, 57, 57f
 - molecular epidemiology for, 60
 - pandemic, 58
 - steps of, 58–59, 58t
 - widespread, 57–58, 58f
 - during pandemic, 52–53
- Infection control (*Continued*)
 - response plan and, 62–63
 - risk assessment, 60
 - surveillance, 53–55, 54b
 - baseline data, 54
 - data gathering and, 54–55
 - definition of, 53–54, 53t
 - general or targeted, 54
- Infection preventionists, 51–52
- Infection rate, 54
- Infection-induced airway obstruction, 795
- Infections *see also specific infections*
 - in special populations, 974, 974b, 982b
- Infectious Diseases Society of America (IDSA), 812
- Infectious mononucleosis, 220, 722
- Infectious substances
 - packaging and shipping of, 98–100
 - shipping of, 128–130, 129f
- Infectious waste, disposal of, 85, 85f, 86f
- Infective endocarditis, bacteremia and, 910
- Inflammation, as host resistance factors, 42, 43f
- Influenza, 223, 392, 808–809
 - meningitis and, 895
 - route of transmission, 33f
- Influenza A virus, 734
- Influenza C virus, 735
- Influenza D virus, 735
- Influenza virus, 717–718, 734
- Ingestion, phagocytosis and, 42
- Inhalation anthrax, 365, 760, 761f, 989
- Inhibition zone, 280–281
- Inhibitors, neutralization of, in cultures, 913
- Inhibitory tests, of *Mycobacterium* spp., 593
- Injectable anthrax, 365–366
- Innate immune response, 975
- Innate immunity, 43, 45f
- Innate resistance, 261
- INNO-LiPA *Mycobacteria* v2 test, 594
- Inoculation, 131–132, 280, 281f
- Inoculum standardization, 276, 276f
- Insect vectors, 662
- Insertion sequence (IS), 251
- In-solution hybridization, 231
- Institutionalized care, UTI in, 926
- Integrans, 268
- Interferon-gamma release assays (IGRAs), 821
- Interferons, 40–41
- Intermediate cycle, 734
- Intermediate hosts, 276–277, 647
- Intermittent bacteremia, 906
- Internal membrane surface, plasma membrane, 8f
- International Standards Organization (ISO), 272–273
- Internodal rhizoids, 616
- Interpretation concerns, of MBC tests, 301
- Intertrigo, 834–836
- Intestinal amebae, 646–647
 - life cycle of, 647–654, 648f
 - treatment of, 647
- Intestinal flukes, 682–683, 683f
- Intestinal parasites, specimens examined for, 643–644
- Intestines, perforation of, 873

Intraabdominal infections, bacteremia and, 910
 Intraocular chambers infections, 1014, 1014b
 Intrauterine devices, 503–504
 Intravascular device, 53
 Intrinsic resistance, 260
 Invader assay, 240–241, 240f
 Invader technology, 240f
 Invasion, 37
 Invasive procedures, increased use of, 907
Iodamoeba bütschlii, 650t, 652, 653f, 654f
 Iodophors, 72
 Ionizing radiation, disinfection and sterilization with, 70
 IQCP. *See* Individualized quality control plan
 Iron uptake, 592, 592f
 Irregular form, of colony morphology, 174–175, 174f
 Isolated colonies, rapid tests on, 194, 194t
 Isolation streak, 132, 134f
 Isolation techniques, for specimen, 132
 Isolator lysis-centrifugation system, 589
 Isolator system, 409
 Isoniazid, 574
 Itraconazole (ITR), 635
Ixodes spp., 672

J

James E. Martin Biological Environmental Chamber (JEMBEC), 375, 375f
 Japanese encephalitis virus, 733
 Jarisch-Herxheimer reaction, 537, 541
 JEMBEC system, 128
 Jenner, Edward, 768
 Jumping genes, 21–22

K

K antigen, 419, 433
 Kanagawa phenomenon, 460
 Kanamycin, 253t–254t
 Kanamycin-vancomycin-laked blood agar (KVLB), 508, 508t, 519f
 Kaposi sarcoma herpesvirus (KSHV), 725, 856, 857f
 Karyosome, 643
 Keratin, 833
 Keratitis, 1002
 amebic, 656
 bacterial, 1009–1010, 1009f, 1010b, 1010f
 fungi and, 1010–1011, 1011b
 parasites and, 1011–1012, 1012f
 viral, 1010, 1010f
 Keratoconjunctivitis, epidemic, 1007
 Ketolides, 259
 Killing, phagocytosis and, 42
 Kinetoplast, 663–664
Kingella denitrificans, 385t, 402–403
Kingella kingae, 403, 403f
Kingella spp., 402–403
 Kinyoun stain, 586, 821
 Kirby-Bauer test, 279
 Kissing bug, 666
Klebsiella, 426–427, 426t
Klebsiella aerogenes, 426, 428t
Klebsiella granulomatis, 962
Klebsiella oxytoca, 427
Klebsiella pneumoniae, 26, 36t, 426, 427f

Klebsiella pneumoniae carbapenemase, 291
Klebsiella pneumoniae subsp. *ozaenae*, 426t, 427
Klebsiella pneumoniae subsp. *pneumoniae*, 426t, 428t
Klebsiella/Enterobacter-like organisms, 172b, 173f
 Klenow fragment, 232–233
Kluyvera spp., 176, 429, 429f
 Koch, Robert, 572
 Köhler, George, 203
 Koplik spots, 737, 737f
 Kovac's reagent, 190, 190f, 396–397
 Krebs cycle, 18, 19f
 Kyasanur Forest disease virus, 733

L

La Crosse virus (LACV), 729
 Labeled immunoassays, 210–219
 Laboratory
 analytic analysis of tests in, 116
 choosing a method in, 119
 clinical analysis of tests in, 116–117
 evaluating and interpreting diagnostic tests in, 116
 investigation support from, 59
 operational analysis of tests in, 117–119
 PCR applications in, 237–238
 performance improvement in, 103, 103b, 120b–121b, 121b
 quality control in, 110–116
 reporting by, 61–62, 61t
 to committees and programs, 61
 to media, 61
 to public health, 61
 role of, in infection control, 50, 51b, 62b–63b, 63b
 safety and, 62
 scientists, in infection control education, 62
 support and data gathering, 55
 test validation in, 119–121
 Laboratory levels or extents of service, 589, 589b
 Laboratory Response Network (LRN), 98, 756–757, 756b, 756f, 757f, 828
 Laboratory safety
 biological safety cabinet and, 82, 83b, 83f, 583
 bioterrorism and, 98–100
 chemical safety and, 85–96
 considerations of miscellaneous safety and, 97
 electrical safety and, 97
 fire safety and, 96–97
 general, 78–98, 78b
 for hazardous chemicals, 96
 hazardous waste and, 85, 85f, 86f
 infectious substances, packaging and shipping of, 98–100
 in microbiology, 77–78
 Mycobacterium spp. and, 583
 processing of patient specimens, 81–82, 82b
 program for, 78–85
 signage for, 96, 96f
 storage of compressed gases and, 97
 training for, 97
 Laboratory safety (*Continued*)
 ventilation and, 583
 in working with actively growing cultures, 82, 82b
 Laboratory-acquired disease, 382
 Laboratory-acquired infections (LAIs), 79
Lacazia loboi, 845
 Lacrimal apparatus infections, 1013
 microorganisms associated with, 1013b
 β -Lactam antibiotics, 253t–254t, 255
 chemical structure of, 256f
 classification schemes for, 257t
 β -Lactam ring, 255, 263f
 β -Lactamases, 262, 319–320
 inhibitors, 266, 266t
 tests, 291–292, 292f
Lactobacillus spp., 504, 524
Lactococcus spp., 339
 Lactoferrin, 37
 Lactophenol cotton blue stain, 12f, 13, 631
 Lactose, 19
 Lactose degradation, 184
 Lactose fermentation, carbohydrate utilization and, 19
 Lactose fermenters, 172, 177b, 184
 Lactose nonfermenters, 172, 172f, 177b
 LAIs. *See* Laboratory-acquired infections
 Lake Victoria Marburg virus, 732–733
 Lancefield classification system, *Streptococcus* spp. and, 340
 Larva migrans, 700–701
 Laryngoscopy, 803
 Lassa viruses, 729
 Latent syphilis, 958
 Lateral flow immunoassays, 216
 Latex agglutination, 207–208, 207f, 208t, 209f, 210t
 tests, 471
 Lean principles, in laboratory, 108
 Learn from defect tool, 109
 Lecithinase, 517, 517f, 521–522
Leclercia, 439
Leclercia adacarboxylata, 439
 Leeches, 464
 Leeuwenhoek, Anton van, 4
Legionella pneumophila, 814
Legionella spp., 407–411, 814, 814f
 antimicrobial susceptibility of, 411
 colony morphology of, 409–410, 410f
 general characteristics of, 407
 identification of, 410–411, 411f, 414b
 infections caused by, 408
 epidemiology of, 408
 isolation of, 135t, 409
 laboratory diagnosis of, 408–409
 microscopic examination of, 409, 409f
 phenotypic characteristics of, 410b
 serologic testing of, 411
 specimen collection and handling of, 409
 virulence factors of, 407–408
 Legionellosis, 408
 Legionnaires' disease, 408
Leishmania braziliensis complex, 663
Leishmania donovani complex, 663
Leishmania mexicana complex, 662–663

- Leishmania* spp., 662–664
 clinical infections of, 662–663
 laboratory diagnosis of, 663–664, 664f
 life cycle of, 663, 664f
- Leishmania tropica* complex, 662–663
- Leishmaniasis, 662, 860–861, 861f
- Lemniscata*, 439
- Lentivirinae, 739
- Leprosy (Hansen disease), 571, 580, 852, 852f
- Leptospira* spp., 534, 534f, 991, 991f
 from blood culture, 916
- Leptospire, 534–538, 534f, 535b
- Leptospirosis, 34t, 534, 850, 991–992, 991b, 992b
- Lesion, specimen collection from, 125t–126t
- Lethal factor, 364
- Leucine aminopeptidase, 335, 342
- Leuconostoc* spp., 339, 339f
- Leukocidins, 35
- Levels or extents of service, laboratory, 589, 589b
- Levine, Phoebus, 19
- Levofloxacin, 253t–254t
- L-forms, 559
- Lichtheimia* spp., 616, 616f
 hyphae, 616
- LightCycler system, 234, 235f
- Lim broth, 14
- Lincomycin, 253t–254t
- Lincosamides, 253t–254t
- Line of identity, 206f
- Linezolid, 253t–254t, 259, 787
- Lipase, 517, 517f
- Lipooligosaccharide, 373
- Lipopeptides, 253t–254t
- Lipopolysaccharide (LPS), 9, 37, 252, 544
- Liposome-mediated agglutination, 209–210, 210t, 211f
- Liposomes, 269
- Liquid media, 588–589
- Listeria monocytogenes*, 354–356
 bacterial meningitis and, 892
 clinical infections of, 355
 colony morphology of, 358f
 diarrhea and, 878
 differentiation of, 357t
 identification of, 356
 laboratory diagnosis of, 356f
 virulence factors of, 355
- Liver flukes, 683–685, 683f
- LJ medium, 587
- Loa loa*, 701–702, 702t
- Lobar pneumonia, 793
- Lobomycosis, 843t, 845
- Local outbreaks, outbreak investigation and, 57, 57f
- Lockjaw, 502
- Loeffler medium, 350
- Louseborne fever, 537
- Louseborne typhus, 554, 554f, 995
- Low pathogenic avian influenza (LPAI), 998
- Lowenstein-Jensen medium, 171t
- Lower respiratory tract infections, 805–823, 806t–807t
- Lower urinary tract infections (L-UTIs), 924, 925b, 931t, 936–937
- LPS. *See* Lipopolysaccharide
- LRN. *See* Laboratory Response Network
- Luminex assay, 716
- Luminometer, 240
- Lung abscess, 816, 818–819
- Lung flukes, 685
- Lupus vulgaris, 851–852
- Lyme borreliosis, 536, 848, 993–994, 993b, 993f
- Lyme disease, 534, 848, 993
Borrelia burgdorferi and, 537
- Lymphadenitis, 573, 961
- Lymphadenopathy, 960f, 961
- Lymphocytes, 45
- Lymphocytic choriomeningitis (LCM) virus, 729
- Lymphocytosis, 804–805
- Lymphogranuloma venereum (LGV), 545, 547f, 945
 clinical manifestations of, 960–961, 961f
 epidemiology of, 960
 laboratory diagnosis of, 961–962
 overview of, 960–962
 treatment of, 962
- Lymphokines, 46–47, 572
- Lysine, 188–189, 189f
- Lysine iron agar (LIA) test, 189f, 190–191, 441t
- Lysogeny, 23
- Lysosomes, 5f, 10, 41
- Lysozyme, 37
- Lyssavirus*, 742
- M**
- M protein, 327
- M11-A8 document, CLSI, 528
- MacConkey agar (MAC), 14, 14f, 171t, 172f, 914
Mycobacterium spp. growth on, 593
 nonfermenting gram-negative bacilli and, 476, 477f
- Macroaspiration, 819b
- Macroconidium, 604–605
- Macrogametocytes, 669
- Macrolides, 253t–254t
- Macrophages, 41
 tissue distribution of, 41t
- Macroscopic examination, 507, 507t
- Macroscopic observation, of specimen, 131
- Mad cow disease, 749
- Madura foot, 843f
- Madurella* spp., 608, 843, 843f
- Magnesium, 232
- Major facilitator superfamily (MFS), 262
- Major outer membrane protein (MOMP), 544
- Malaria, 667–668, 897
 laboratory diagnosis of, 670
 life cycle of, 669, 670f
 route of transmission, 33f
 species comparisons of, 671t
 treatment of, 668
- Malassezia furfur*, 603, 603f, 837
- MALDI Biotyper, 481
- MALDI-TOF. *See* Matrix-assisted laser desorption/ionization-time-of-flight
- Malignancy, 977–979
- Malignant pustule, 365
- Mansonella ozzardi*, 702–703, 702t
- Mansonella perstans*, 702–703, 702t
- Mansonella* spp., 702–703, 702t
- Mansonella streptocerca*, 702–703, 702t
- Manual lysis centrifugation system (Isolator), 916
- Manual multitest systems, 192–193, 192t
- Marburg disease, 765
- Marburg virus, 732, 764–766
- Marcolide resistance, 322
- Margin, colony morphology and, 174–175
- Martin-Lewis medium, 376t
- Mass spectrometry
 MALDI-TOF, 246, 246f
Mycobacterium spp. and, 594
- Mastadenovirus*, 718–719
- Matrix protein (M), 734
- Matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF), 246, 309, 321b, 476, 520–521
 mass spectrometry, 184, 186t, 187f, 197, 379
 bacteremia and, 918, 919f
- Maurer dots, 671–672
- MB/BacT Alert 3D System, 588–589
- McFarland turbidity standards, 275–276
- Measles virus, 737, 853
 route of transmission, 33f
- mecA* gene, 287
- Media
 agar-based, 587
 colony morphology and, 170–173
 cornea storage, 1021–1022
 for diarrhea, 883, 885t
 egg-based, 587
 inoculated, anaerobic incubation of, 510–512
 laboratory reporting to, 61
 liquid, 588–589
Mycobacterium spp. and, 587–589, 588t, 589b
 plated and tubed, inoculation of, anaerobic specimens and, 508–510
 procedure of, 509–510
 quality control of, 111–113, 112f, 113f, 114t
- Median infectious dose (ID₅₀), 871
- Mediastinitis, 761, 761f
- Medical materials, disinfection and sterilization and, classifications of, 69, 69t
- Mediterranean spotted fever, 850, 995
- Medusa head morphology, 174–175, 366
- Medusa-head colonies, 761, 762f
- Melioidosis, 487, 762–763
- Melting curve analysis, 234
- Melting temperature (T_m), 229
- Membrane channel protein, plasma membrane, 8f
- Membrane filters, 787
- Membrane-bound immunoassays, 216–217, 216f
- Meningismus, 895
- Meningitis, 891–898
Aeromonas spp. and, 464
 aseptic, 893
 bacterial, 891–893, 891b, 892f, 893f
Brevundimonas spp. and, 489–490
 direct antigen detection assay for, 223
Haemophilus influenzae causing, 393

- Meningitis (*Continued*)
herpesvirus and, 895
human immunodeficiency virus and, 895
influenza and, 895
Neisseria meningitidis causing, 381
Pseudomonas oryzoilhabitans and, 484
tuberculosis and, 573
tuberculous, 895
viral infections and, 893–895, 893*b*, 893*f*, 894*b*, 894*f*, 901
- Meningococemia, 381, 381*f*
- Meningoencephalitis, 894, 898–899. *See also* Encephalitis
- Merozoites, 678
- Merthiolate-iodine-formalin (MIF), for fecal samples, 641*t*
- Mesophiles, 15
- Mesosome, bacteria, 5*f*
- Messenger RNA (mRNA), 21, 252
- Metabolism
bacterial, 16
biofilms and, 781
- Metacercaria, 682
- Metachromatic granules, 7
- Metagenomics, 244, 247
- Metagonimus yokogawai*, 683, 684*t*
- Methicillin-resistant *Staphylococcus aureus* (MRSA), 312, 320, 787, 840–841
susceptibility testing and, 287
- Methicillin-resistant *Staphylococcus epidermidis* (MRSE), 320
- Methicillin-susceptible *Staphylococcus aureus* (MSSA), 315
- Methyl red (MR) test, 187, 188*f*
- Methylene blue stain, 12*f*, 13, 564–565
- Methylobacterium* spp., 491
- Metronidazole, 952
- MGIT 960 system, 588
- MIC. *See* Minimal inhibitory concentration
- Microaerophiles, 132–134
- Microaerophilic bacteria, 15
- Microaerophilic environment, 467
- Microaerophilic organism, 497, 497*t*
- Microaspiration, 819*b*
- Microbes, frequently identified, in infection control, 55–56
- Microbial biota
composition of
at different body sites, 27–30
factors that determine, 27
origin of, 26–27
role of
in host defense against infectious disease, 30–31
in pathogenesis of infectious disease, 30
- Microbial infections, changing paradigm of, 784–785
- Microbial load, 67
- Microbial morphotypes, 148–149
- Microbiology laboratory
analytic analysis of tests in, 116
choosing a method in, 119, 120*f*
clinical analysis of tests in, 116–117
evaluating and interpreting diagnostic tests in, 116
operational analysis of tests in, 117–119, 119*b*
- Microbiology laboratory (*Continued*)
performance improvement in, 103, 103*b*, 120*b*–121*b*, 121*b*
benchmarking and, 109–110
continuous education and training, 106
customer concept and, 109
establishing performance monitors, 107–108, 108*b*
fixing the process, 109
indicators of, 106–107
Lean and Six Sigma, 108
learn from defect tool and, 109
mission, vision and values statement and, 105
personnel competency and proficiency assessments and, 105–106
problem-action form for, 108, 109*t*
quality control in, 110–116
antimicrobial susceptibility and, 114–115, 115*t*
equipment maintenance and, 110–111, 110*t*
manual for, 115
media and, 111–113, 112*f*, 113*f*, 114*t*
reagents and, 113, 114*f*
temperature and, 110
thermometer calibration and, 110–111
use of stock cultures, 115
test validation in, 119–121
- Microbiology laboratory safety, 77–78
- Microbiota, in large intestine, 870*t*
- Microcephaly, 734
- Micrococcus* spp., 309, 310*f*
- Microconidium, 604–605
- Microfilariae, 644, 701, 702*t*, 703*f*
- Microimmunofluorescence (MIF), 552
- Microorganisms
associated with conjunctivitis, 1005*b*
blepharitis and associated with, 1008*b*
classification/taxonomy of, 6–7
control of, 65–66, 66*b*, 98*b*, 99*b*–100*b*
degree of killing, factors influencing, 67–69
biofilms, 68
compatibility of disinfectants, 68–69
concentration of disinfecting agent, 68
contact time, 68
nature of surface to be disinfected, 68
number of organisms, 67, 68*f*
pH, 68
presence of organic material, 68
temperature, 68
types of organisms, 67, 67*f*
overview of, 4–6
pathogenic, 793, 794*b*
planktonic, 777
search for, 148–149, 148*f*
skin entry of, 39, 40*t*
- MicroScan Rapid Anaerobe Identification Panel, 520
- MicroScan systems, 196, 196*f*
- MicroScan WalkAway SI, 294–295, 294*f*
- Microscopes, 143, 144*t*
testing frequency of, 110*t*
- Microscopic examinations, 139–168, 139*b*–140*b*, 146*b*, 152*b*
characterization of background materials in, 146–148, 147*f*
- Microscopic examinations (*Continued*)
classification of materials in, 149–151, 149*t*
contamination and, 149–150, 149*f*
examination of prepared materials in, 144–149
examples of sample observation in, 153
of fungi, 631
grading of materials in, 149–151, 149*t*
initiation of special handling for
unsuspected or special pathogens, 152, 152*f*
local materials and, 150–151, 150*f*, 151*f*
microscopes and, 143, 144*t*
mixed materials and, 151
purulence and, 151
quality control in, 153
reports of, 151–152, 152*b*
sample preparation and, 140–142, 140*t*
search for microorganisms in, 148*f*
staining and, 142–143
terminology of, 143–144, 144*t*–145*t*, 145*t*
- Microscopic morphology
of *Campylobacter* species, 469, 469*f*
of *Vibrio* spp., 456–457, 457*f*
- Microscopic observation, of specimen, 131
- Microscopy, for viral infections, 715
- MicroSeq Microbial Identification System, 521
- Microsporidia, 643, 681–682, 681*f*, 682*f*, 1011–1012, 1012*f*
diarrhea and, 880, 880*b*
- Microsporium audouinii*, 606, 606*f*
- Microsporium canis*, 605–606, 606*f*
- Microtiter plate, 787
- Middle East respiratory syndrome (MERS), 809–810
- Middle East respiratory syndrome coronavirus (MERS-CoV), 710, 731
- Middlebrook 7H11 medium, 587
- Miescher, Frederick, 19
- MIF. *See* Merthiolate-iodine-formalin
- Miliary tuberculosis, 573
- Milker nodule, 857
- Milwaukee protocol, 742
- Minimal biofilm elimination concentration, 787
- Minimal inhibitory concentration (MIC), 272–273
agar dilution MIC test, 279, 279*f*
breakpoint (cutoff), 278
broth macrodilution MIC tests, 277
broth microdilution MIC tests, 277–279, 277*f*, 278*f*, 278*t*
tube dilution test, 277
- Minimum bactericidal concentration (MBC), 300–301, 300*t*, 301*b*, 301*f*
- Minocycline, 253*t*–254*t*, 787
- Miracidium, 682
- Miscellaneous gram-negative bacilli, 474, 474*b*–475*b*, 493*b*, 493*b*–494*b*
- Missense mutation, 22
- Mission statement, 105
- Mitochondria, 5*f*, 10
- Mitsubishi Gas Chemical America, 506
- Mixed acid fermentation, 17, 185
- Mobile genetic elements, 21–22
- Mobiluncus* spp., 503

- Modified acid-fast stain, 643
 Modified polyvinyl alcohol, for fecal samples, 641*t*
 Modified Robbins cell, 787
 Modified Thayer-Martin medium, 376*t*
 Moeller decarboxylase base medium, 189
Moellerella, 439
Moellerella wisconsensis, 439
 Moist heat, 69–70
 Molds, 4–6, 598, 852–853
 test for identification of, 632
 yeast *versus*, 598–599
 Molecular beacons, 235–236, 236*f*
 Molecular diagnostics
 applications of, 227, 228*b*
 clinical microbiology laboratory, 243–247
 Molecular epidemiology, outbreak investigation and, 60
 Molecular methods, 646
 Molecular probes
 concentration, 229
 degree of target and probe complementarity, 229
 length of, 229
 salt concentration of, 229
 selection of, 229–230
 Mollicutes, 559, 559*t*, 563*t*
 Molluscum contagiosum, 856, 857*f*, 971, 971*f*
 Monkeypox virus, 710, 727
 Monobactams, 255, 256*t*
 Monoclonal antibodies, 203, 203*f*
 Monocytes, 41
 tissue distribution of, 41*t*
 Monograph system, 75
 Monomicrobial infections, 146, 146*f*
 Mononucleosis, 220, 722
Moraxella catarrhalis, 383–384, 488, 793
 characteristics of, 385*t*
 clinical infections of, 383, 383*f*
 laboratory diagnosis of, 383–384
 specimen collection and identification of, 383–384
Moraxella nonliquefaciens, 488
Moraxella osloensis, 488
Moraxella spp., 488
 Morbillivirus, 736
Morganella, 431, 431*t*
Morganella morganii, 431
 Morganellaceae, 430–431
 Morphology, of bacteria, 11–13
 microscopic shapes, 11, 11*f*
 Morulae, 555, 996
 Mosquitoes, 733
 Motility organelles, in eukaryotes, 11
 Motility test, 191, 515
 for *Listeria monocytogenes*, 357*f*
 Motility-indole-ornithine (MIO) agar, 191
 Mouth, normal microbiota of, 28–29, 28*b*
 Moxifloxacin, 253*t*–254*t*
 MRSA. *See* Methicillin-resistant *Staphylococcus aureus*
 MRSE. *See* Methicillin-resistant *Staphylococcus epidermidis*
 MSSA. *See* Methicillin-susceptible *Staphylococcus aureus*
 Mucocutaneous leishmaniasis, 663
Mucor spp., 616, 616*f*
 Mucorales, 598–599, 599*f*, 601, 616–617, 844
 Mucormycosis, 601, 844
 Mueller-Hinton agar, 279, 463, 528
 Mueller-Hinton broth, 277
 Multidrug and toxic effects (MATE) family, 262
 Multidrug resistance (MDR), 251
 Multidrug-resistant tuberculosis, 574
 Multilocus enzyme electrophoresis (MLEE), 242
 Multilocus sequence typing (MLST), 243
 Multilocus variable number of tandem repeat analysis (MLVA), 243
 Multiplex PCR, 232–233, 237
 Multispecies biofilm, 777
 Multispot HIV-1/2 rapid test, 741
 Mumps virus, 736–737, 894–895
 route of transmission, 33*f*
 Mupirocin, 837
 Murein, 8
 Murine typhus, 553–554, 995
 Murray-Washington method, 149
 Musculoskeletal infections, bacteremia and, 910
 Mutations, 22
 Mutualism, 26
 Mycelia, 4–6, 598
 Mycetoma, 607–608, 843, 843*f*, 843*t*
 actinomycotic, 361
 eumycotic, 361
Mycobacteria, 36*t*
 Mycobacterial growth indicator tube (MGIT) system, 588
 Mycobacterial infection, 846–847, 895, 901–902
 skin infections from, 851–852
Mycobacterium, 363*t*
Mycobacterium africanum, 581*t*–582*t*
Mycobacterium asiaticum, 581*t*–582*t*
Mycobacterium avium complex, 575–576, 581*t*–582*t*
Mycobacterium avium subsp. *paratuberculosis*, 576
Mycobacterium bovis, 575, 581*t*–582*t*
Mycobacterium chelonae, skin infections from, 851*f*
Mycobacterium chelonae-Mycobacterium abscessus group, 579, 581*t*–582*t*
Mycobacterium fallax, 581*t*–582*t*
Mycobacterium fortuitum group, 579, 581*t*–582*t*
Mycobacterium gastri, 581*t*–582*t*
Mycobacterium genavense, 577
Mycobacterium haemophilum, 577, 581*t*–582*t*
Mycobacterium kansasii, 576–577, 577*f*, 581*t*–582*t*
Mycobacterium leprae, 580, 580*f*, 852
Mycobacterium mageritense, 581*t*–582*t*
Mycobacterium marinum, 577, 577*f*, 581*t*–582*t*
Mycobacterium peregrinum, 581*t*–582*t*
Mycobacterium scrofulaceum, 577–578, 578*f*, 581*t*–582*t*
Mycobacterium simiae, 578, 581*t*–582*t*
Mycobacterium smegmatis group, 579–580
Mycobacterium spp.
 biochemical identification of, 590–593, 592*f*, 593*f*
 blood and, 585
 from blood culture, 917
 chromatography and, 593
Mycobacterium spp. (Continued)
 colony morphology and, 589
 culture media and isolation methods for, 587–589, 588*t*, 589*b*
 disinfectants and, 583
 general characteristics of, 571–572
 Gram staining of, 586, 587*t*
 growth rate and, 589–590
 inhibitory tests of, 593
 isolation and identification of, 580–594, 581*t*–582*t*
 laboratory identification of, 589–594, 589*b*
 laboratory safety considerations for, 583
 MacConkey agar (MAC) growth of, 593
 photoreactivity of, 590, 591*b*
 specimens and, 583–585, 584*b*
 stool and, 585
 temperature and, 590
 urine and, 584–585
 usual clinical significance of, 571*b*
Mycobacterium szulgai, 578, 581*t*–582*t*
Mycobacterium terrae complex, 581*t*–582*t*
Mycobacterium triviale, 581*t*–582*t*
Mycobacterium tuberculosis, 15, 570, 572–575, 581*t*–582*t*, 759
 case on, 570*b*–571*b*
 diagnosis of, 573–574, 574*f*
 extrapulmonary tuberculosis and, 573
 hybridization and nucleic acid amplification tests for, 594
 multidrug-resistant, 574–575
 nodular lymphangitis and, 847
 primary tuberculosis and, 572–573
 reactivation tuberculosis and, 573
 skin infections and, 851–852
 susceptibility testing of, 594–595
Mycobacterium tuberculosis complex (MTBC), 572–575
Mycobacterium ulcerans, 578, 581*t*–582*t*
Mycobacterium xenopi, 578–579, 581*t*–582*t*
 Mycolic acid, 9
Mycoplasma fermentans, 562
Mycoplasma genitalium, 561–562
Mycoplasma hominis, 559, 561–562, 563*t*
Mycoplasma pneumoniae, 559–561, 561*f*
Mycoplasma salivarium, 566*f*
Mycoplasma spp., 558, 558*b*
 antimicrobial susceptibility for, 567
 clinical infections with, 560–562
 culture and, 563–566, 564*t*
 general characteristics of, 559–560, 559*t*, 560*f*, 561*t*
 indigenous to humans, 561*t*
 isolation and identification of, 564–566, 566*b*, 566*f*
 laboratory diagnosis of, 562–567
 media and, 564, 565*f*
 nucleic acid amplification tests for, 563
 result interpretation of, 567–568, 568*t*
 serologic diagnosis of, 566–567, 567*t*
 specimen collection and transport for, 562–563
 Mycoses, 601–603, 601*f*
 cutaneous, 602, 602*t*
 opportunistic, 615–622
 subcutaneous, 602
 superficial, 602
 systemic, 602

- Myonecrosis, 503, 503b
Myotis occultus, 731
Myroides spp., 490
- N**
- Naegleria fowleri*, 655–656, 656f
 Nails, 626
 Nalidixic acid, 253t–254t
Nannizzia gypsea, 606, 606f
 Nanoarrays, 245
 Nanobiotechnology, 244
 Nanoencapsulation, 268
 Nanomedicine, 247–248
 Nanoshells, 269
 Nasopharyngeal aspirates, 413
 Nasopharyngeal carcinoma (NPC), 723
 Nasopharynx
 biota of, 793
 flora of, 793b
 normal microbiota of, 28–29, 29b
 specimen collection from, 125t–126t
 National Committee for Clinical Laboratory Standards (NCCLS), 319–320
 National Fire Protection Association (NFPA), 85–86, 86f
 Natural antibodies, 793
 NCCLS. *See* National Committee for Clinical Laboratory Standards
 NDA. *See* New drug application
Necator americanus, 695
 Necrotizing fasciitis, 328–329, 842, 842b
 Necrotizing soft tissue infection, 842–843, 842f
 Negative predictive value (NPV), clinical applications of, 117–118
 Negri bodies, 715
 Neighbor-joining (NJ) method, 244
Neisseria bacilliformis, 385t
Neisseria cinerea, 384, 385t, 387f
Neisseria elongata, 385t, 387–388
Neisseria flavescens, 385t
Neisseria gonorrhoeae, 15, 171–172, 374–380, 380b, 945, 947f. *See also* Gonorrhea
 antimicrobial resistance of, 380
 carbohydrate utilization for, 378
 characteristics of, 385t
 colony morphology of, 376–377, 376f, 377f
 culture of, 375
 direct microscopic examination of, 375, 376f
 identification of, 376–377, 378t, 379t
 incubation of, 376
 infections
 clinical, 374–375, 375b
 epidemiology of, 374
 treatment of, 380
 isolation of, 376t
 laboratory diagnosis of, 375–380
 microscopic morphology of, 377, 377f
 nucleic acid amplification tests for, 379–380, 379t, 380t
 oxidase disk test for, 377–378, 377f
 penicillinase-producing, 380
 specimen collection and transport of, 375, 375f
Neisseria lactamica, 385t, 387, 387f
Neisseria meningitidis, 27, 380
 characteristics of, 385t
 direct microscopic examination of, 381, 382f
 identification of, 382, 382f
 infection
 clinical, 381
 epidemiology of, 381
 laboratory-acquired, 382
 treatment of, 382–383
 vaccine for, 383
 isolation of, 376t
 laboratory diagnosis of, 381–382
 specimen collection and transport of, 381
Neisseria mucosa, 385t, 387
Neisseria polysaccharea, 385t
Neisseria sicca, 385t, 387, 387f
Neisseria spp., 371, 372b
 colony morphology of, 385t
 commensal, 384–388, 384t, 386t
 general characteristics of, 372f, 373, 373t, 385t
 identification of, 384, 386t
 pathogenic, 373–384
 virulence factors of, 373, 374f
Neisseria subflava, 387, 388f
Neisseria subflava biovars, 385t
Neisseria weaveri, 385t, 388
Neobalantidium coli, 657
 Neonatal herpes, 720, 956
 Neonates
 Chlamydia trachomatis and, 547, 547t
 density of bacteremia in adults *versus*, 911–912
 genital mollicutes and, 562, 563t
 immunocompromised patients and, 981–982
 Neoplasia, 780
 Nephropathia epidemica, 729–730
 Nested PCR, 232–233, 237
 Neuraminidase inhibitors, 736
 Neurosyphilis, 540
 Neutralization assays, 210
 Neutropenia, 825–826, 977
 Neutrophils, 825–826
 New drug application (NDA), 75
 New World arenaviruses, 729
 New World hantaviruses, 730
 New York City medium, 376t
 Newborn, listeriosis in, 355
 Next-generation sequencing, 245
 NF. *See* Necrotizing fasciitis
 NFPA. *See* National Fire Protection Association
 Niacin test, 591
 Nicotinamide adenine dinucleotide (NAD), 17
 9/11 Commission Report, 771–772
 Nitrate disk, 516
 Nitrate reduction test, 191, 591–592, 592f
Nocardia brasiliensis, 361
Nocardia spp., 360–362, 363t
 characteristics of, 360–361, 360f
 clinical infections of, 361
 colony morphology of, 362f
 isolation of, 135t
 laboratory diagnosis of, 361–362
 virulence factors of, 361
 Nocardioform, term, 363
 Nocardiosis, 846
 treatment of, 362
 Nocobactin, 361
 Nodular lymphangitis, 845–847
 actinomycosis and, 847, 847f
 causes of, 845b
 mycobacterial infection, 846–847
 Mycobacterium tuberculosis and, 847
 nocardiosis, 846
 nontuberculous mycobacteria and, 846–847
 sporotrichosis, 845–846, 845f
 Nomenclature, 6
 Non-A, non-B (NANB) hepatitis, 747
 Nonchromogens, 576
 Noncritical materials, disinfection and sterilization and, 69
 Nonfermenting gram-negative bacilli, 474–475, 474b–475b, 493b, 493b–494b
 biochemical characteristics and identification of, 475–476, 476b, 477f, 478b, 478t, 479f
 carbohydrates and, 475
 clinical infections from, 475, 475b
 clinically significant, 476–488
 general characteristics of, 475–476
 less commonly encountered, 488–494
 MAC and, 476, 477f
 taxonomic changes for, 476, 478t
 Nongonococcal urethritis (NGU), 546–547, 561–562, 945
 Nonhemolytic organisms, 173
 Nonhemolytic streptococci, 326t
 Nonionizing radiation, disinfection and sterilization with, 70
 Nonlactose fermenters (NLFs), 184, 440
 Nonoxidizers, 475
 Nonpathogenic intestinal flagellates, 662
 Nonpolar region of phospholipid, plasma membrane, 8f
 Nonroutine specimens, 136, 136b
 Nonselective media, 14, 131
 Nonsense mutation, 22
 Nonsusceptible organisms, 276–277
 Nontreponemal antibody tests, 958
 Nontuberculous mycobacteria (NTM), 570
 case on, 570b–571b
 clinical significance of, 575–580
 nodular lymphangitis and, 846–847
 pneumonia and, 820
 rapidly growing species of, 579–580, 591f
 skin infections and, 851–852
 slowly growing species of, 575–579, 590f
 Nontypable *Haemophilus influenzae*, 392
 Norfloxacin, 253t–254t
 Normal biota, of respiratory tract, 793–794, 793b
Norovirus spp., 34
 Noroviruses (NoVs), 730, 730f
 North American blastomycosis, 612
 Northern blot, 231
 Norwalk-like virus, 875
 Norwegian scabies, 861, 862f
 Nose, normal microbiota of, 29b
 Nosocomial infections, 310
 Novobiocin susceptibility test, 318, 319f
 NPV. *See* Negative predictive value

- Nuclear envelope, 5f
 5' nuclease assay, 234–237, 235f
 Nucleic acid amplification
 bdDNA detection for, 239, 240f
 hybrid capture for, 240, 240f
 NASBA for, 238–239, 238f
 procedures of, 231–241
 TMA for, 239
 Nucleic acid amplification tests (NAATs), 522, 918, 918f, 948
 for *Chlamydia psittaci*, 551–552, 552b
 Mycobacterium tuberculosis and, 594
 Neisseria gonorrhoeae and, 379–380, 379t, 380t
 ocular infections and, 1007, 1019
 tuberculosis and, 821
 Nucleic acid detection, for *Bordetella*, 413
 Nucleic acid hybridization
 applications of, 231
 formats, 230–231
 in-solution, 231
 in situ hybridization, 231
 Mycobacterium tuberculosis and, 594
 overview and techniques of, 228–231, 228f
 reaction variables, 229
 solid support, 230
 Nucleic acid probes, 344–345
 Nucleic acid sequence-based amplification (NASBA), 238–239, 238f, 716
 Nucleocapsid, 710
 Nucleolus, 5f, 10
 Nucleoprotein (NP), 734
 Nucleoside reverse transcriptase inhibitors (NRTIs), 741–742
 Nucleus, 5f
 Nugent scoring system, 359, 360t
 Numeric codes, 192
 Nutritional resources, 778
 Nutritionally variant streptococci, 338, 916
 Nutritive media, 14
 Nymphs, 993
- O**
 O antigen, 421, 433
 Obligate aerobes, 15, 497t, 498
 Obligate anaerobes, 15, 497t, 498
 Obligate intracellular parasites, 6, 710–712
 Occult (unsuspected) bacteremia, 905
 Occupational Safety and Health Administration (OSHA), 78
 Ocular herpes, 720
 Ocular infections, 1001, 1001b–1002b, 1021b
 biofilm-centered, 1014–1016, 1015t, 1016f
 chlamydial, 1006–1007, 1006f
 of cornea, 1009–1012
 culture, 1017–1019, 1018t, 1020f
 direct microscopic examination and, 1016–1017, 1017f, 1017t
 endophthalmitis, 1002, 1014, 1014b
 episclera and, 1012
 of eyelids, 1008–1009
 general concepts related to, 1002–1004
 HIV and, 1014
 host protective mechanisms and, 1003
 of intraocular chambers, 1014, 1014b
 laboratory diagnosis of, 1016–1022
 of lacrimal apparatus, 1013, 1013b
 microbial biota, 1002–1003, 1003b, 1003t
 microorganisms associated with, 1004b
 ocular infections (Continued)
 NAATs and, 1007
 ocular structures and, 1002, 1002f
 of orbit, 1012–1013
 sclera and, 1012
 specimen collection for, 1016, 1016f
 virulence factors of pathogenic organisms of, 1003–1004, 1004b
 Ocular larva migrans, 701
 Ocular medications, contaminated, 1020, 1020b
 Odor, colony morphology and, 177
 Odynophagia, 803
 O/F basal medium (OFBM), 185, 185f, 186t
 Old World arenaviruses, 729
Oligella spp., 488
Oligella urethralis, 488
 Oligonucleotide primers, 228f, 240–241
Onchocerca volvulus, 702, 702t, 703f, 859, 860f
 Onchocercosis, 702
 Oncosphere, 687–688
 Oncovirinae, 739
 Onychomycosis, 605
 Opaque density, 175, 175f
 Operational analysis, of tests, 117–119
 Operations benchmarking, 109–110
 Ophthalmia neonatorum, 375
 Ophthalmic drops, 1020
Opisthorchis felineus, 684
Opisthorchis spp., 684
Opisthorchis viverrini, 684
 Opportunistic infection, 27, 794
 Opportunistic mycoses, 615–622
 Opportunistic pathogens, 29, 31, 31t
 Coccidioides immitis and, 821–822
 endogenous, 975
 exogenous, 975
 immunocompromised patients and, 974–975, 975t
 respiratory tract infections and, 823
 Opportunists, 30
 Opsonins, 40, 817
 Opsonization, 35, 41–42
 Optical immunoassay (OIA), 217, 217f
 Optochin susceptibility, 331t, 344, 344f
 Oral anthrax, 760–761
 Oral cavity, normal microbiota of, 28
 Oral herpes, 720
 OraQuick Advance rapid HIV-1/2 antibody test, 741
 OraQuick in Home HIV Test, 966
 OraQuick test, 217f
 Orbit infections, 1012–1013
 Orbital cellulitis, 1012
 sinusitis and, 800
 Orf virus, 857
 Organ culture, 716
 Organ transplantation, 979–980
 Organic material, presence of, disinfection and, 68
Orientia spp., 552, 554–555
Orientia tsutsugamushi (scrub typhus), 995
 Oritavancin, 253t–254t
 Ornithine, 188–189
 Oropharyngeal infections, 374–375
 Oropharynx
 biota of, 793
 flora of, 793b
 normal microbiota of, 28–29, 29b
 Orthomyxoviridae, 734–736
 Ortho-nitrophenyl- β -D-galactopyranoside (ONPG) test, 187, 187f, 194t
Orthopoxvirus, 767
 Orthostatic changes, 873
 Oseltamivir (Tamiflu), 736
 OSHA. See Occupational Safety and Health Administration
 Osteomyelitis, 314
 OTC. See Over-the-counter
 Otitis externa, 481
 Otitis media, 795, 797t, 801–802
 causes of, 801
 clinical manifestations of, 802
 complications with, 802
 epidemiology of, 801
 laboratory diagnosis of, 802
 pathogenesis of, 801–802
 treatment for, 802
 Ouchterlony gel diffusion, 206, 206f
 Outbreak investigations, 56–62, 57b, 57f
 antibiograms and, 59, 59t
 cultures and serology for, 59–60
 environmental culturing and, 60–61, 60b
 air, 60
 water, 60–61
 laboratory support for, 59
 local, 57, 57f
 molecular epidemiology for, 60
 in pandemic, 58
 steps of, 58–59, 58t
 widespread, 57–58, 58f
 Outbreaks, 53, 56–57
 Outcome monitors, 107
 Outer membrane, bacteria, 5f
 Over-the-counter (OTC) drug, 72
 Oxacillin, 253t–254t, 277t, 287–288, 288b, 288f
 screen plate, 287
 Oxalic acid, 586
 Oxazolidinones, 253t–254t, 259
 Oxidase disk test, 377–378, 377f
 Oxidase test, 191, 194t
 Oxidation, 185
 Oxidation-fermentation tests, 185–186, 185f, 186t, 316
 Oxidative-fermentative media (OF media), 475
 Oxidizers, 475
 Oxid Signal system, 914
- P**
 Palivizumab (PVZ), 738
 PAMPs. See Pathogen-associated molecular patterns
 Pandemic, 709–710
 outbreak investigations during, 56–58
 Pandemic preparedness, 771–772
Pantoea, 430
Pantoea agglomerans, 430, 430f
 Pantone-Valentine leukocidin (PVL), 35, 311, 812
 Papanicolaou (Pap) smears, 715
 Papillomas, 726
 Papillomaviridae, 726
 Papules, 835b
 Parachlorometaxylenol (PCMX), 74
Paracoccidioides spp., 600, 615, 615f

- Paracoccus* spp., 490
Paracoccus yeei, 490
 Para-dimethylaminobenzaldehyde (PDAB), 190, 190f
 Paradoxical (Eagle) effect, 301
 Paragonimiasis, 685
Paragonimus kellicotti, 685
Paragonimus westermani, 685, 685f, 897
 Parainfluenza virus, 736
 Paramyxoviridae, 736–738
 mumps virus in, 736–737
 parainfluenza virus in, 736
Paramyxovirus, 736
 Parapneumonic effusions (PPEs), 813
 Parasites, 4, 29
 blood and tissue, examination of
 specimens for, 644–645
 case on, 640b
 conjunctivitis and, 1008
 diarrhea and, 879–881, 879b
 endophthalmitis and, 1014, 1015f
 fecal specimens and, 640–643
 immunologic diagnosis of, 645–646
 intestinal and urogenital parasites, other
 specimens examined for, 643–644
 keratitis and, 1011–1012, 1012f
 medically important, 646–706, 647t
 obligate intracellular, viruses as, 710–712
 quality assurance and, 646
 Parasitic infections, 858–861
 in central nervous system, 896–898,
 902–903, 902b, 902f
 Parasitism, 26
 Parasitology, diagnostic, 639, 640b, 704b,
 705b–706b
 general concepts in, laboratory methods
 of, 640–646
 blood and tissue parasites and, 644–645
 fecal specimens and, 640–643, 641t
 immunologic diagnosis and, 645–646
 intestinal and urogenital parasites and,
 643–644
 quality assurance in, 646
 medically important parasitic agents in,
 646–706, 647t
 Apicomplexa, 667–681
 blood and tissue roundworm
 infections, 700–706, 700f
 cestodes, 691–692
 flukes, 682–687, 683f, 684t
 helminths, 682
 hookworms, 695–697, 697b, 697f, 698f
 microsporidia, 681–682
 protozoa, 646–667, 662b
 roundworms, 692–695, 693t
 Strongyloides stercoralis, 697–699, 698f,
 699f, 700f
 tapeworms, 687–691, 687f, 688t
 Parinaud oculoglandular conjunctivitis, 545
 Paronychia, 840
 Paroxysmal phase, of pertussis, 412
 Parrot fever, 548
 Parvoviridae, 727–728
 Parvovirus B19 infection, 854
 Parvovirus B19 viremia, 728
Pasteurella, 404, 405t
Pasteurella multocida, 404, 404f
 Pasteurellosis, 404, 986, 986b
 Pasteurization, 70
 Pathogen-associated molecular patterns
 (PAMPs), 43
 Pathogenesis, 31
 ability to produce extracellular toxins and
 enzymes and, 37–39
 ability to survive intracellularly and
 proliferate and, 37
 adhesion to host cells and tissue in,
 35–37, 36f
 endotoxins and, 39, 39t
 exotoxins and, 37, 38t, 39, 39t
 microbial factors contributing to, 31–39
 resistance to phagocytosis and, 33t, 35,
 36t
 role of microbiota in, 30
 routes of transmission, 32–35, 33f, 33t
 Pathogenic bacteria, 7
 Pathogenic intestinal flagellates, 658–662,
 658f, 659t
 Pathogenic microorganisms, 793
 Pathogenic urogenital flagellates, 658–662,
 659t
 Pathogenicity, 31, 780–782, 781b
 Pathogens, 26
 emerging and reemerging, 51, 51b
 Patient preoperative skin preparation, 75,
 76t
 Patient-collected specimens, 124–126
 Pattern recognition receptors (PRRs), 43
 Paul-Bunnell heterophile antibody test,
 723–724
 PBP. *See* Penicillin-binding protein
Pediococcus spp., 339–340
 Pelvic inflammatory disease (PID), 374,
 546–547, 945, 970–971
 Penems, 255
 Penetration, viral replication and, 712
 Penicillin, group B streptococci and,
 330–332, 345
 Penicillinase-producing *Neisseria*
 gonorrhoeae (PPNG), 286, 380
 Penicillinase-resistant penicillins, 281
 Penicillin-binding protein (PBP), 255, 321
 β-lactam antibiotics and, 255
 Penicillins, 256t, 541, 567, 959
 disk-diffusion testing and, 281
 overview of, 253t–254t
 for *Streptococcus pneumoniae*, 284–285
Penicillium spp., 620, 620f
Pentatrachomonas (*Trichomonas*) *hominis*, 662
 Pentose phosphate pathway, 17, 18b, 18f
 Peptidoglycan, 5f
 biosynthesis of, 252
 overview of, 253t–254t
 structure of, 256f
Peptostreptococcus spp., 504
 Peracetic acid, hydrogen peroxide and, 74
 Performance improvement (PI), in
 microbiology laboratory, 102,
 120b–121b, 121b
 benchmarking and, 109–110
 continuous education and training and,
 106
 customer concepts and, 109
 establishing performance monitors and,
 107–108, 108b
 fixing the process in, 109
 Performance improvement (PI), in
 microbiology laboratory (*Continued*)
 indicators of, 106–107
 Lean and Six Sigma and, 108
 learn from defect tool and, 109
 mission, vision and values statement and,
 105
 personnel competency and laboratory
 proficiency assessments and, 105–106
 problem-action form for, 108, 109t
 quality and, measures of, 105–110
 Perihepatitis, 374
 Perinatal period, 982
 Periodontitis, 782–783
 Aggregatibacter actinomycetemcomitans
 causing, 399–400, 401f
 Peripheral chromatin, 643
 Periplasmic flagella, 534
 Periplasmic space, bacteria, 5f, 9
 Peritonitis, 484
 Permanently stained smears, 643
 Peroxisome, 5f, 10
 Persistent diarrhea, 872
 Persistent effect, chloroxylenol, 74
 Persister cells, 260, 301, 779
 Personal protective equipment (PPE), 583,
 758–759
 Personnel competency, assessment of,
 105–106, 107f
 Personnel protective equipment (PPE), 79,
 80f
 Pertussis, 411, 797t, 804–805
 catarrhal phase of, 412
 causes of, 804
 clinical manifestations of, 412, 804
 complications with, 804
 convalescent phase of, 412
 epidemiology of, 412, 804
 laboratory diagnosis of, 804–805
 paroxysmal phase of, 412
 pathogenesis of, 804
 treatment for, 805
 Pertussis toxin (PT), 412
 Petechiae, 835b, 849
 Petragani medium, 587
 PFGE. *See* Pulsed-field gel electrophoresis
 pH
 gastric, 870
 nucleic acid hybridization reactions and,
 229
 sterilization and disinfection and, 68
 Phaeohyphomycosis, 844
 Phaeoid, hyaline *versus*, 599–600, 599f
 Phaeoid yeast, 622
 Phage typing, 7
 Phagocytes, 35
 Phagocytosis, 41f, 41t
 attachment and, 41–42
 as host resistance factors, 41–42
 ingestion and, 42
 killing and, 42
 resistance to, 33t, 35, 36t
 Phagosome, 37, 42, 42f
 Pharyngitis, 328, 547–548, 793, 796–799
 Arcanobacterium haemolyticum causing,
 358
 case study on, 796b, 797b
 causes of, 796, 797t

- Pharyngitis (*Continued*)
 clinical manifestations of, 797
 complications with, 798
 epidemiology of, 796
 group A streptococcal pharyngitis, 796, 798
 laboratory diagnosis of, 798, 798f
Neisseria gonorrhoeae causing, 374–375
 pathogenesis of, 796
 streptococcal, direct antigen detection assay for, 222–223, 222t
 treatment for, 798–799
- Phenicol, 253t–254t
- Phenol, 66
- Phenolics, 73–74
- Phenotype, 6, 184
 classification by, 7
- Phenylalanine deaminase (PAD), 189, 189f
- Phenylethyl alcohol (PEA), 366, 508, 508t
- Pheromones, 779
- Phialides, 600
- Phialoconidia, 600, 600f
- Phialophora verrucosa*, 607t
- Phoma* spp., 622, 622f
- Phospholipid bilayer, plasma membrane, 8f
- Photochromogens, 576, 577f
- Photoreactivity, of *Mycobacterium* spp., 590, 591b
- Photosynthesis, 10
- Phyla, 6
- Physical methods, of disinfection and sterilization, 69–70
 filtration, 70
 heat, 69–70, 70t
 radiation, 70
- Picornaviridae, 738–739, 738b
- Piedraia hortae*, 603
- Pigment, colony morphology and, 176
- Pili, 5f, 10, 35
- Pinta, 538, 541
- Pinworm, 693
 cellophane tape preparation for, 643
- Pithomyces* spp., 622, 622f
- PKDL. *See* Post-kala-azar dermal leishmaniasis
- Plague, 992–993
 bubonic, 769, 992
 cause of, 992
 clinical manifestation of, 992
 life cycle to, 992
 pneumonic, 757, 828, 992
 septicemic, 770, 770f
- “Plague of Justinian,” 769
- Planktonic forms, 777
- Planktonic phenotype, 778
- Plaques
 dental, 782–783
 verrucous, 835b
- Plasma membrane
 cholesterol in, 11
 of eukaryotes, 11
 of prokaryotes, 8, 8f
- Plasmid profile analysis, 241
- Plasmid-mediated quinolone resistance (PMQR), 267
- Plasmids, 21, 241, 260
- Plasmodium falciparum*, 667–668, 671–672, 671t, 673f
- Plasmodium knowlesi*, 667–668
- Plasmodium malariae*, 667–668, 671, 671t, 672f
- Plasmodium ovale*, 667–668, 671, 671t, 673f
- Plasmodium* spp., 667–672
 clinical infections of, 668
 laboratory diagnosis of, 670–672, 671t
 life cycle of, 669f, 670f
 treatment of, 668–669
- Plasmodium vivax*, 667–668, 671, 671t, 672f
- Plate reading, 171–172
- Pleocytosis, 891
- Pleomorphic, defined, 9
- Pleomorphic gram-positive bacilli, 350
- Plesiomonas shigelloides*, 429, 443–452
- Plesiomonas* spp., 429–430
 key features of, 458t
- Pleuropneumonia-like organism (PPLO), 559
- Pneumocystis jirovecii*, 224, 224f, 625, 625f, 965–966, 966f
- Pneumocystis* spp., 624–626, 625f, 626f
- Pneumonia, 547–548, 808. *See also*
 Aspiration pneumonia; Community-acquired pneumonia; Hospital-acquired pneumonia; Ventilator-associated pneumonia
 acute, 811–817
Aspergillus spp. and, 821–822
 bacteremia and, 910
 chronic, 819–823
Cryptococcus spp. and, 821–822
 fungi and, 821–822
Legionella causing, 408
Mycoplasma pneumoniae and, 560–561
 NTM and, 820
 primary atypical, 560–561
Pseudomonas aeruginosa and, 481
 tuberculosis and, 818b, 819–823, 820b
- Pneumonic plague, 437, 757, 828, 992
- Pneumonitis, 361
- Pneumovirus*, 736
- Point mutation, 22
- Polar region of phospholipid, plasma membrane, 8f
- Poliovirus, 738
- Polyclonal antibodies, 203, 203f
- Polymerase chain reaction (PCR), 232–238, 233b, 234b, 580–583
 contamination prevention and, 233
 digital, 237
 electrophoresis and, 230
 laboratory applications of, 237–238
 multiplex, 232–233, 237
Mycobacterium tuberculosis identification and, 594
 nested, 232–233, 237
 product analysis of, 233–237
 real-time, 233–234, 234f
 repetitive palindromic extragenic elements, 242–243
 reverse transcription, 232–233, 237
 steps in, 232t
- Polymicrobial bacteremia, 906
- Polymicrobial infections, 146, 146f
 direct examination of, 167, 167f, 168f
- Polymorphic fungi, 600
- Polymorphism, 600
- Polymorphonuclear cells, 35, 781
- Polymyxin B, 253t–254t
- Polymyxin E (colistin), 253t–254t
- Polypeptides, 253t–254t
- Polyvinyl alcohol (PVA), 641
- Pontiac fever, 408
- Population studies, 205
- Porin proteins, bacteria, 5f
- Porins, 261–262
- Porphyrin test, 396–397, 397f
- Porphyromonas* spp., 504, 526
- Positive predictive value (PPV), 932
 clinical applications of, 117–118
- Postanalytical activity, 104–105
- Post-analytical phase, of workflow, in laboratory, 104–105, 104f
- Post-kala-azar dermal leishmaniasis (PKDL), 663
- Postsplenectomy, 980, 980b
- Poststreptococcal sequelae, 329
- Postzone, 208
- Potassium hydroxide
 with calcofluor white, 629, 629f
 preparation of, 628–629, 628f
- Potassium nitrate assimilation, 634
- Pott disease, 573
- Poxviridae, 726–727
- Poxviruses, 726–727
- PPE. *See* Personnel protective equipment
- PPV. *See* Positive predictive value
- Prenatal activity, 104–105
- Pre-analytical phase, of workflow, in laboratory, 104, 104f
- Precautions
 standard, 62, 78–79
 transmission-based, 79
- Precision, 116
- Predictive values, of tests, 117–118
- Pregnancy
 immunocompromised patients and, 981–982
 listeriosis during, 355
 UTI and, 927
- Prereduced, anaerobically sterilized media (PRAS media), 506, 508–509, 509f
- Preseptal cellulitis, 1012
- Preservatives, specimen and, 128
- Presurgical skin disinfection, 77
- Prevalence, 55
 of disease, 117
- Preventive maintenance program, 111, 111f
- Prevotella melaninogenica*, 176
- Prevotella* spp., 504, 524–526
- Primary amebic meningoencephalitis (PAM), 896–897
- Primary atypical pneumonia, 560–561
- Primary bacteremia, 906
- Primary cell cultures, 716
- Primary infection, 53, 53f
- Primary inoculation, of specimen, 131–132
- Primary plating, 170
- Primary pyoderma, 837
- Primary tuberculosis, 572–573
- α -Prime, 326t
- Primer annealing, 232
- Primer extension, 232, 232t
- Primer-dimers, 237
- Primers, 232

- Prions, 67, 749
 Pristinamycin, 253t–254t
 Probe, 229
 Probe amplification reactions, 239
 Probe-based assays, 231
 Probe-mediated stains, 142–143, 143t
 ProbeTec, 551–552
 Problem-action form, 108, 109t
 Process monitors, 106
 Proctitis, 545, 960–961, 971
 Proficiency testing (PT), 106
 Proglottids, 687, 687f
 Prokaryotes
 cell structure of, 7–10
 cell envelope in, 8–10
 cytoplasmic, 7–8
 surface polymers in, 10, 10b
 classification by, 7
 eukaryotes and, 4, 4t–5t, 5f
 Promastigote, 663
Propionibacterium spp., 27, 524, 524f
 Propionic acid fermentation, 17
 Prostatic secretions, 937
 Prostatitis, 925b, 931
 Proteases, 517
 Protective antigen, 364
 Protein, plasma membrane, 8f
 Protein A, 35, 312
 Protein expression, 21
 Proteolytic enzymes, 517
 Proteolytic reactions, 517
 Proteomics, 244, 246–247
Proteus mirabilis, 430, 430f
Proteus spp., 174–175, 175f, 430–431, 431t
 Protozoa, 4, 646–667, 662b
 central nervous system infections and, 896–897, 897f
Providencia, 431, 431t
 Prozone, 208
Pseudallescheria boydii, 843, 843t
 Pseudobacteremia, 905
 Pseudo-germ tubes, 632, 633f
 Pseudohyphae, 623, 633–634, 634f
 Pseudomembranous colitis, 503
 Pseudomonads, 476–478, 483
Pseudomonas aeruginosa, 36t, 38t, 481–483, 481f, 482b
 bacteremia and, 481
 identifying characteristics of, 482, 482f
 nutritional requirement for growth, 14
 pigment production and, 176, 176f
 pneumonia and, 481
 treatment for infections of, 482–483
 virulence factors of, 481–482, 482f, 482t
Pseudomonas alcaligenes, 484
Pseudomonas bacteremia, 847–848
Pseudomonas fluorescens, 483, 483b
Pseudomonas luteola, 484
Pseudomonas mendocina, 484
Pseudomonas oryzihabitans, 484, 484f
Pseudomonas pseudoalcaligenes, 484
Pseudomonas putida, 483, 483b
Pseudomonas spp., 476–478, 847–848
 fluorescent group, 481–483
 nonfluorescent group, 483–484
 scum produced by, 177–178, 178f
Pseudomonas stutzeri, 483, 483f
 Pseudopodia, 4
 Psittacosis, 548
Psychrobacter spp., 488
 Psychrophiles, 15
 Public health
 laboratory reporting to, 61–62, 61t
 settings
 frequently identified microbes in, 55–56, 56t
 infection control in, 52, 52t
 Puffballs, 177–178, 178f
 Pulmonary anthrax, 989
 Pulmonary infections, *Nocardia* causing, 361
 Pulsed-field gel electrophoresis (PFGE), 60, 242, 242f
 Purified protein derivative (PPD), 572, 572b, 821
 Purine, 19
 Purpura, 835b
Purpura fulminans, 841
Purpureocillium lilacinum, 619
Purpureocillium spp., 619, 620f
 Purulence, 140, 140f
 evidence of, 146–147, 147f
 Pustules, 835b
 Putrescine, 188–189
 PVA. *See* Polyvinyl alcohol
 PVL. *See* Panton-Valentine leukocidin
 Pyelonephritis, 923–924, 925b, 934–936
 Pyocyanin, 482
 Pyoderma, 837–841, 838t
 Pyodermal infections, 328
 Pyogenic streptococci, 326–327
 Pyoverdin, 482
 Pyrazinamidase test, 593, 593f
 Pyrazinamide (PZA), 574
 Pyrimidine, 19
 Pyrophosphate (PPi), 245
 Pyrosequencing, 244–245
 Pyrrolidonyl aminopeptidase, 341–342
 Pyrrolidonyl arylamidase activity (PYR), 316
 Pyrrolidonyl- α -naphthylamide (PYR)
 hydrolysis, 329–330, 331t, 341–342, 343f
 Pyruvic acid
 aerobic utilization of, 18, 19f
 anaerobic utilization of, 17–18
 biochemical pathways from glucose to, 17, 17f
 Pyuria, 932, 935
- Q**
 Q fever, 556
 QA. *See* Quality assurance
 QBC. *See* Quantitative buffy coat
 QC. *See* Quality control
qnr genes, 267
 Q-probes, 110
 Quality assurance, 103
 in parasitology laboratory, 646
 Quality benchmarking, 110
 Quality control
 for antimicrobial susceptibility testing, 295–298, 296t, 297t, 298t
 in microbiology laboratory, 103, 110–116
 antimicrobial susceptibility and, 114–115, 115t
- Quality control (*Continued*)
 equipment maintenance and, 110–111, 110t
 manual for, 115
 of media, 111–113, 112f, 113f, 114t
 of reagents, testing methods and, 113, 114f
 temperature and, 110
 thermometer calibration and, 110–111
 use of stock cultures and, 115
 in microscopic examinations, 153
 Quality management system
 introduction to, 103–105
 workflow and, 103–105, 104f
 Quantitative buffy coat (QBC), 645–646
 Quantitative isolation technique, 132, 134f
 Quaternary ammonium compounds, 73
 Quinolone resistance-determining region (QRDR), 264
 Quinolones, 258
 Quinupristin, 259–260
 Quinupristin-dalfopristin, 253t–254t
 Quorum sensing (QS), 779
 Quorum-quenching compounds, 786–787
 Quorum-sensing inhibitors (QSIs), 786–787
- R**
 RabAvert, 742
 Rabies virus, 6, 715, 742
 route of transmission, 33f
 RACE acronym, in fire safety, 96–97
 Racquet hyphae, 598–599, 599f
 Radial immunodiffusion, single, 206
 Radiation, disinfection and sterilization with, 70
 Radioimmunoassay (RIA), 219
 RADTs. *See* Rapid antigen detection tests
 Ragpicker's disease, 365, 760
Rahnella spp., 439
Ralstonia pickettii, 492
Ralstonia spp., 492
 Random amplified polymorphic DNA (RAPD), 242–243
Raoultella, 427
 Rapid and semiautomated identification systems, *Vibrio* spp. and, 461
 Rapid antigen detection tests (RADTs), 798
 group A streptococci pharyngitis and, 330
 Rapid carbohydrate degradation tests, 378
 Rapid CB Plus system, 354
 Rapid diagnostic tests, 666–667
 Rapid method, 193
 Rapid modified Wright-Giemsa stain, 142–143, 143t
 Rapid plasma reagin (RPR) test, 219–220, 220f, 540
 Rapid systems, 193–194, 194t
 evaluation of, 197
 Rapid tests, 514–517
 RapID-ANA II, 520, 520f
 RASE. *See* Recognized as safe and effective
 Rashes, 835b
 Rat bite fever, 850–851
 Rats, 992
Rattus rattus, 769
 Rayon, 713
 RDTs. *See* Rapid diagnostic tests
 Reactivation tuberculosis, 573

- Reading plates, 280–281, 281*f*, 282*f*, 282*t*, 283*t*–284*t*
- Reagents, quality control of, testing methods and, 113, 114*f*
- Reagin antibodies, 206
- Reagin screen test (RST), 958
- Real-time PCR, 233–234, 234*f*
- Recognized as safe and effective (RASE), 75
- Recurrent cellulitis, 839
- Red blood cells (RBCs), hemolysis and, 326
- Reemerging pathogens, 51
- Regan-Lowe medium, 413
- Reiter syndrome, 546–547
- Relapsing fever, 534
- Relative light units (RLUs), 230
- Relenza, 736
- Renal transplantation, 927
- Reoviridae, 728–729, 875
- Repetitive palindromic extragenic elements PCR, 242–243
- Replication, DNA interference, 258
- Reporters, 245
- Reporting
by laboratory, 61–62, 61*t*
susceptibility testing results of, 274–275, 275*b*
- Resident biota, 76
- Resident microbiota, 27
- Resistance
acquired, 260
mechanisms of, 263–266
antimicrobial
bacteremia and, 907–908
mechanisms of, 260–266, 260*b*
origins of, 260–261
bacterial mechanisms, 250*b*–251*b*
biofilms and, 261
dissemination of, 267–268, 267*b*
efflux pumps and, 262*f*, 263–264
enzymatic inactivation and, 262–263, 263*f*
enzymatic target site alteration and, 264–265
impermeability and, 261–262
induced, 261
innate, 261
intrinsic mechanisms of, 261–263
new target acquisition and, 265
target site modification and, 264–265
- Resistance modulation cell division superfamily (RND), 262
- Respiration, 16–17
- Respiratory burst, 42
- Respiratory diphtheria, 349–350
- Respiratory specimens, mycotic sample collection of, 628
- Respiratory syncytial virus (RSV), 715, 737–738, 801
- Respiratory tract
anaerobes and, 499–500
anatomy of, 792, 792*f*
cleansing mechanism of, 40
normal biota of, 793–794, 793*b*
normal microbiota of, 28–29, 28*b*
specimen collection from, 125*t*–126*t*
- Respiratory tract infections, 790, 828*b*
age and, 794, 811*t*
Aspergillus spp. and, 826
barriers to, 792–793
- Respiratory tract infections (*Continued*)
bioterrorism and, 827–829
case on, 792
empiric antimicrobial therapy and, 795
general concepts of, 792–794
host defense evasion and, 796
host risk factors and, 794–795
in immunocompromised host, 823–827
infection-induced airway obstruction and, 795
lower, 805–823, 806*t*–807*t*
pathogenic microorganisms
distinguished from normal biota in, 793–794, 794*b*
reduced clearance of secretions and, 794–795
seasonal and community trends in, 795
toxin elaboration and, 795
upper, 796–805, 797*t*
viral, 223
emerging, 809–811
virulence factors and, 795–796, 796*b*
- Response plans, emergency, in infection control, 62–63
- Reston ebolavirus (RESTV), 732, 765
- Restriction enzymes, 23–24, 242
- Reticulate body (RB), 544
- Retinitis, 1014
- Retroorbital abscess, sinusitis and, 800
- Retroviridae, 739–742, 740*b*
- Reveal G2 rapid HIV-1 antibody test, 741
- Reverse passive agglutination, 206–207
- Reverse transcription-polymerase chain reaction (RT-PCR), 232–233, 237, 730
- Rhabditiform larva, 693*t*, 697
- Rhabdomyosarcoma (RD) cells, 717
- Rhabdoviridae, 742
- Rhadinovirus* spp., 725
- Rheumatic fever, 329, 798
- Rheumatoid factor (RF), 202
- Rhinocerebral mucormycosis, 616
- Rhinocladia aquaspersa*, 607*t*
- Rhinoscleroma, 427
- Rhinopodidiosis, 843*t*
- Rhinoviruses, 739
- Rhizoid form, of colony morphology, 174–175, 174*f*
- Rhizoids, 598–599, 599*f*
- Rhizopus* spp., 616, 616*f*, 617*f*
- Rhodococcus*, 363–364, 363*t*
- Rhodococcus equi*, 363
- Rhodotorula* spp., 624
- Ribavirin, 738
- Ribonuclease H (RNase H), 238–239
- Ribonucleic acid (RNA)
anatomy of, 19–21
double-stranded, 728–729
Northern blot for, 231
ribosomal, 7, 259
single-stranded, 729–743
- Ribosomal mutation, 264
- Ribosomal ribonucleic acid (rRNA), 7, 259
- Ribosome, 5*f*
bacterial, 7
eukaryotic, 10
- Ribotyping, 241
- Rice grains, growth on, 632
- Rickettsia akari*, 552
- Rickettsia conorii*, 552
- Rickettsia prowazekii*, 552
- Rickettsia* spp., 543*b*–544*b*, 552–554
comparative properties of, 545*t*
general characteristics of, 994
laboratory diagnosis of, 554–555
overview of, 994–996
spotted fever group, 553, 994–995
transitional group of, 554
typhus group of, 553–554
- Rickettsia typhi*, 552
- Rickettsialpox, 554, 994
- Rickettsiosis, 849–850
- Rifampin, 253*t*–254*t*, 258, 574, 787
- Rifamycins, 253*t*–254*t*, 258
- Rifapentine, 253*t*–254*t*
- Rift Valley fever virus, 729
- Ringworm, 34*t*
- Risk assessment, 54
biological, 79–81
- Risk groups, 759
for infectious agents, 81, 81*b*
- Risus sardonicus, 502
- Ritter disease, 311
- River blindness, 702
- Roche Molecular Systems, 948*t*
- Rocky Mountain spotted fever (RMSF), 553, 553*f*, 849, 898, 994, 994*f*
- Rolling circle amplification (RCA), 239
- Roseolovirus* spp., 725
- Roseomonas* spp., 491, 492*f*
- Rotaviruses, 728, 875
- Rothia*, 354
- Rothia aerea*, 354
- Rothia dentocariosa*, 354
- Rothia mucilaginosa*, 354
- Rough endoplasmic reticulum, 5*f*, 10
- Roundworms, 692–695, 693*t*
- Rubella, 220, 743, 853–854
route of transmission, 33*f*
- Rubeola, 853
- Rubivirus*, 742–743
- Rubulavirus*, 736
- ## S
- Sabouraud dextrose agar (SDA), 171*t*, 491, 1017, 1019*f*
- SAF. *See* Sodium acetate-acetic acid-formalin
- Safety, in laboratory, 62
- Safety data sheets (SDSs), 86–87, 87*f*
- Saline, 713
- Salmonella* spp., 36*t*, 432–435
antigenic structures in, 433, 434*f*
bacteremia from, 435
as bioterror agents, 755
carrier state of, 435
classification of, 433, 433*t*
clinical infections from, 433–435
diarrhea and, 876, 884, 884*f*, 885*t*
enteric fever and, 434–435, 876
food poisoning, 876
gastroenteritis and, 434, 876
serologic grouping in, 442–443, 452*t*
virulence factors of, 433
- Salmonella typhi*, 10
- Salmonella typhimurium*, 38*t*
- Salmonella-Shigella* (SS) agar, 440
- Salmonellosis, 433–434

- Salpingitis, 546–547
Salt concentration, 229
Salt tolerance test, 343
Sample preparation, 140–142, 140t
Sanger, Frederick, 244
Sanger sequencing, 244–245
Sapoviruses (SaVs), 730
Saprobes, 598, 615
Saprophyte, 615
SARS-associated coronavirus (SARS-CoV), 731
Satellitism, 392, 392f
Scalded skin syndrome (SSS), 310, 313
Scarlet fever, 328, 854, 864
Scedosporium boydii, 608, 609f
Schaeffer-Fulton stain, 8
Schistosoma haematobium, 684t, 685, 687f
Schistosoma japonicum, 684t, 685, 687f
Schistosoma mansoni, 684t, 685, 687f
Schistosoma spp., 685
 clinical infections of, 685–686
 laboratory diagnosis of, 686–687, 687f
 life cycle of, 686, 686f
Schistosomiasis, 685, 859
Schizogony, 669, 669f
Schizont, 669
Schüffner stippling, 671
Sclera, 1012
Scleritis, 1012
Scolex, 687, 687f
Scombroid, 881
Scopulariopsis spp., 620, 620f
Scorpion primer, 236, 236f
Scotochromogens, 576, 578
Scrub typhus, 850, 995–996
Scum, 177–178, 178f
Scutula, 605
SDSs. *See* Safety data sheets
Seasonal trends, in respiratory tract infections, 795
Secondary bacteremia, 906
Secondary immune responses, 201–202, 202f
Secretions, reduced clearance of, 794–795
Select agents, 756
Select biological agents, 404
Selective media, 14, 131
Selective reporting protocol, 274–275, 275b
Semicritical materials, disinfection and sterilization and, 69, 69t
Semi-quantitative catalase test, 592, 592f
SEN virus, 743–744, 748–749
Sensititre Automated Incubator Reader, 295, 295f
Sensititre System, 195t
Sentinel laboratories, 98
Sepsis, 904, 904b–905b, 907b, 913b, 920b
 biomarkers for, 918–919, 919b
 definitions of, 905–906, 905t
 mortality of, 906–907
 severe, 905t
 treatment of, 919–920
Septate hyphae, 598–599, 599f
Septic shock, 905, 905t
Septicemia, 905
 Aeromonas spp. and, 464
 Erysipelothrix rhusiopathiae causing, 357
 mortality of, 905
 neutropenia and, 977
 Vibrio vulnificus and, 460
Septicemic plague, 437, 770, 770f
Septi-Chek, 626–628
Sequencing, 244
Seroconversion, 202
Serologic testing, 200
 agglutination assays, 206–210, 207f, 209f, 210f, 210t, 211f
 antibodies in, 201–205, 201t, 202f, 203f, 204f, 205f
 antigen detection and, 205, 205f
 antigens and, 201
 assessment questions, 225
 of *Bordetella*, 413–414
 of *Borrelia burgdorferi*, 538
 case in point, 200b
 Chlamydia trachomatis detection with, 552
 commercial, 202
 for congenital infections, 205
 of EBV, 723–724, 723t, 724f
 false-negative, 203
 false-positive, 200
 of fungal infections, 221–222
 for HIV, 221
 immune status testing, 205
 labeled immunoassays and, 210–219
 of *Legionella*, 411
 of leptospires, 536
 neutralization assays, 210
 outbreak investigations and, 59–60
 points to remember, 224–225
 population studies, 205
 precipitation assays, 206
 principles of, 206–219
 in specific diseases, 219–222
 for streptococcal infections, 220
 of syphilis, 219–220, 220f
 microscopic examination of, 540
 of *Treponema pallidum*, 540
 Vibrio spp. and, 461–463
 of viral disease, 220–221
 of viruses, 718
Serotyping, 184
Serovarieties, 7
Serratia, 432
Serratia marcescens, 176, 432, 432f
 pigment production and, 177f
Serratia odorifera, 432
Serratia rubidaea, 432, 432f
Serum bactericidal test (SBT), 300t, 302
Sessile phenotype, 777
Severe acute respiratory syndrome (SARS), 731, 809–810
Severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2), 21, 858, 875
Sexual phase, 670
Sexual reproduction, 667
 of fungi, 600, 600f
Sexually transmitted diseases (STDs), 944t
 case study, 968
 causative agents of, 943t
 causing gastrointestinal infection, 879
 definition of, 943–944
Sexually transmitted infections, 927, 942, 943b
 acquired immunodeficiency syndrome, 963–967
 cervicitis, 948–949
Sexually transmitted infections (*Continued*)
 epididymitis, 971
 genital ulcer disease, 953–963
 genital warts, 968–969
 molluscum contagiosum, 971
 pelvic inflammatory disease, 970–971
 proctitis, 971
 urethritis, 945–948
 viral hepatitis, 969–970, 970f
 vulvovaginitis, 949–953
Shared values, 105
Shear stress, 778
Sheep blood agar (SBA), 14, 15f, 171t, 172f, 460–461, 771f
Shell vial culture, centrifugation-enhanced, 718
Sherlock Microbial Identification System, 195t, 247, 247f, 520
Shewanella spp., 492
Shiga toxin (Stx), 424
Shiga toxin-producing *Escherichia coli* (STEC), 424–425
Shigella, 32–34, 38t, 435–439
 antigenic structures of, 436
 clinical infections from, 436–437
 diarrhea and, 876, 884f
 differentiation of, 436t
 serologic grouping in, 443–452
Shigella dysentery, route of transmission, 33f
Shigella sonnei, 435–436, 436f
Shigellosis, 436
Shingles, 855
Shunt infections, 893
Sigmoidoscopy specimens, 643–644
Signage, for laboratory safety, 96, 96f
Signal amplification reactions, 239–241
Silica gel, 510
Simple stains, 142–143, 143t
Simplexvirus, 720
Sin Nombre virus (SNV), 730
Single radial immunodiffusion, 206
Single-stranded deoxyribonucleic acid (ssDNA), 727–728
Single-stranded ribonucleic acid (ssRNA), 729–743
Single-vial systems, for fecal samples, 641t
Sinusitis, 797t, 799–801
 acute, 799
 case study on, 799b, 800b
 causes of, 799
 clinical manifestations of, 799–800, 800f
 complications with, 800
 epidemiology of, 799
 laboratory diagnosis of, 800–801
 pathogenesis of, 799
 treatment for, 801
Six Sigma (6σ) principle, in laboratory, 108
16S ribosomal RNA gene sequencing, 521
Skin
 anaerobes and, 499
 anatomy of, 833–834, 834f
 as barrier against infection, 39, 40t
 biota, 833
 cancer patients and, 978
 normal microbiota of, 27–28, 28b
 SSS and, 310, 313
 Staphylococcus aureus and, 310, 313

- Skin and soft tissue infections, 832, 833*b*, 865*b*, 866*b*
 bacteremia and, 910
 bacterial, 847–852
 bite infections, 841
 diabetic foot infections, 841–842, 842*b*, 842*f*
 fungi causing, 844–845, 852–853
 immune- or toxin-mediated, 861–864
 laboratory diagnosis for, 864–866
 localized, 834–847, 835*b*, 835*t*, 836*t*
 necrotizing, 842–843, 864*b*
 parasitic infections and, 858–861
 viral infections and, 853–858
- Skin snip, 702
- Skipped wells, 278–279
- Sleeping sickness, 664, 665*f*
- Slide agglutination test, 344, 344*f*
- Slide coagulase test, 309
- Slide cultures, 631–632
- Small colony variants (SCVs), 309
- Small intestine, 870–871
 microorganisms in, 29
- Smallpox, 727, 727*f*, 768, 768*f*
 clinical manifestations of, 769–771
 diagnosis of, 769
- Smears
 blood, 644–645
 ocular infections and, 1016, 1017*t*
 Papanicolaou, 715
 permanently stained, 643
 from swab, 141, 141*f*
 from thick, granular, or mucoid materials, 141, 142*f*
 from thick liquids or semisolid, 141, 141*f*
 from thin fluids, 141, 142*f*
 Tzanck, 715
- Smooth endoplasmic reticulum, 5*f*, 10
- Smooth form, of colony morphology, 174–175, 174*f*
- Sodium acetate-acetic acid-formalin (SAF), for fecal samples, 641*t*
- Sodium amylosulfate (SAS), 914
- Sodium chloride tolerance, 593
- Sodium dodecyl sulfate, 218
- Sodium hydroxide, 586
- Sodium hypochlorite, 72–73, 583
- Sodium polyanethol sulfonate (SPS), 128, 911
 disk, 516
- Solid support hybridization, 230
- Solid-phase enzyme immunoassay, 214*f*
- Solid-phase immunosorbent assay, 213*f*
- Southern blotting, 230, 230*f*, 241
- Spanish flu, 735
- Sparganosis, 692
- Special potency antimicrobial disks, 515–516, 517*t*
- Species, 6
- Specificity, 201
- Specimens, 123–124, 124*b*, 137*b*, 137*b*–138*b*
 anaerobe
 acceptable, 505*t*
 selection, collection, transport, and processing of, 505–512, 505*b*, 505*t*
 unacceptable, 505*b*
 anticoagulants and, 128
 biopsy, 645
- Specimens (*Continued*)
 collection of, 123, 127*b*, 932–934, 937*b*
 bacteremia and, 911–913, 911*b*
 basic principles of, 124–127
 catheterized, 933, 937
 fundamentals and, 124
 labeling and requisitions of, 126–127
 for ocular infections, 1016, 1016*f*
 by patient, 124–126
 procedures, 124, 125*t*–126*t*
 safety of, 127
 suprapubic aspiration for, 933, 937
 voided midstream, 933
 communication of laboratory findings in, 136–138
 decontamination and digestion agents for, 585–586
 fecal, 640–643
 holding or transport of, 128, 129*b*
 incubation of, 132–134
 isolation techniques for, 132, 134*f*
 macroscopic observations of, 131
 microscopic observations of, 131
Mycobacterium spp. and, 583–585, 584*b*
 mycology laboratory and, 626, 627*f*, 627*t*
 nonroutine, 136, 136*b*
 preparation of, 132
 preservation of, 128
 primary inoculation of, 131–132
 prioritization of, levels of, 130, 130*t*
 receipt and processing of, 130–134, 134*b*
 rejection, criteria for, 936
 sigmoidoscopy, 643–644
 storage of, 127–128, 128*t*
 suboptimal, 130
 unacceptable, 130–131
 urine, vaginal, and urethral, of parasites, 644
 viral infections and
 for maximum recovery, 713–714, 714*t*
 selection, collection and transport of, 713
- Spectinomycin, 259
- Sphingobacterium* spp., 490
- Sphingomonas paucimobilis*, 492, 493*f*
- Sphingomonas* spp., 492–494
- Spiral hyphae, 598–599, 599*f*
- Spiral staircase, 19–21
- Spirochetal infections, central nervous system and, 895, 902
- Spirochetes, 11, 11*f*
Borrelia spp. and, 536
Leptospira spp. and, 534, 534*f*
 overview of, 533, 533*b*, 541*b*
 treponemes and, 538–541, 538*f*
 virulence factors of, 534, 536
- Splinters consistency, of colony, 176
- Sporangiophores, 601
- Sporangiospores, 601, 601*f*
- Spores, 8, 1012
- Sporicidal activity, 69
- Sporoblast, 679
- Sporocysts, 679
- Sporodochia, 619
- Sporogony, 669, 669*f*
- Sporothrix schenckii*, 609–610, 610*f*
- Sporothrix* spp., 600
- Sporotrichosis, 602, 845*f*
- Sporozoites, 668
- Spot indole test, 194*t*, 515
- Spot tests, 220
- Spotted fever, 553, 553*f*
- Spotted fever group, *Rickettsia* spp., 994–995
- Sputum, 149, 549, 584, 644
 specimen collection from, 126
- SSIs. *See* Surgical site infections
- SSS. *See* Scalded skin syndrome
- St. Louis encephalitis (SLE), 894
 virus, 733
- Staining, microscopic examination and, 142–143, 143*t*
- “Standard microbiological practices,” 759
- Standard precautions, 62, 78–79
- Staphaurex test, 318
- Staphylocoagulase, 318
- Staphylococcal cassette chromosome *mec* (SCC*mec*), 265
- Staphylococcal coagglutination, 208, 209*f*, 210*t*
- Staphylococcal scalded skin syndrome, 863
- Staphylococci, 307, 309*b*
 clinically significant species of, 310–315, 311*t*
 cultural characteristics of, 316, 316*f*
 general characteristics of, 309–310, 310*t*
 identification methods of, 316–318
 identification of, 319*t*, 320*f*
 isolation and identification of, 315–319
 laboratory diagnosis, 315–319
Micrococcus spp. *versus*, 309, 310*f*, 310*t*
 microscopic examination of, 315, 315*f*
 rapid identification methods, 318–319
 specimen collection and handling for, 315
- Staphylococcus aureus*, 6, 36*t*, 38*t*, 181*f*, 310–314
 cultural characteristics of, 316, 316*f*
 cytolytic toxins and, 311
 enterotoxins and, 310–311
 enzymes and, 311–312
 epidemiology of, 312
 exfoliative toxin and, 311
 food poisoning and, 313–314
 infections caused by, 312–314
 as opportunistic pathogens, 29
 oxacillin resistance in, 287
 protein A and, 312
 significance of, 311*t*
 skin and wound infections with, 313
 TSST-1 and, 311
 vancomycin resistance in, 288–289
 virulence factors of, 310–312, 312*t*
- Staphylococcus delphini*, 309
- Staphylococcus epidermidis*, 28, 311*t*, 314, 929, 929*t*, 930*t*
- Staphylococcus hyicus*, 309, 311*t*
- Staphylococcus intermedius*, 309, 311*t*
- Staphylococcus lugdunensis*, 309, 311*t*, 314
- Staphylococcus lutrae*, 309
- Staphylococcus pseudintermedius*, 309, 315*b*
- Staphylococcus saprophyticus*, 311*t*, 314, 929–930, 929*b*, 930*t*
- Staphylococcus schleiferi*, 309
- Staphylococcus sciuri*, 311*t*
- Staphylococcus simulans*, 311*t*
- Steer’s replicator, 279*f*

- Stenotrophomonas maltophilia*, 485–486
 clinical infections from, 485–486
 identifying characteristics of, 486
- Sterilization, 66
 biofilms and, 68
 compatibility of disinfectants in, 68–69
 concentration of disinfecting agent and, 68
 contact time and, 68
 degree of killing in, factors influencing, 67–69
 disinfection *versus*, 66–67
 methods of, 69–71
 chemical, 70
 physical, 69–70
 nature of surface for, 68
 organic material and, presence of, 68
 pH and, 68
 temperature and, 68
- Stock cultures, use of, in quality control, 115
- Stock solutions, antimicrobial, 277
- Stomach, microorganism in, 29
- Stool
 Mycobacterium spp. and, 585
 specimen collection from, 126
- Stop codons, 22
- Strain typing and identification, 241–243
- Strains, 6
- Strand displacement amplification (SDA), 239
- Strawberry cervix, 951, 951f
- Streamers, 177–178, 178f, 778
- Streptobacillus moniliformis*, isolation of, 135t
- Streptococcal pyrogenic exotoxins, 328
- Streptococcal toxic shock syndrome (TSS), 329
- Streptococcus agalactiae*, 180f, 330–332, 892
 antigenic structure of, 330
 classification of, 327t
 clinical infections of, 330–332
 laboratory diagnosis of, 332, 332f
 virulence factors of, 330
- Streptococcus anginosus*
 characteristics of, 336t
 classification of, 327t
- Streptococcus bovis*
 characteristics of, 336t
 classification of, 327t
- Streptococcus dysgalactiae*, classification of, 327t
- Streptococcus equi*, classification of, 327t
- Streptococcus mitis*
 characteristics of, 336t
 classification of, 327t
- Streptococcus mutans*
 characteristics of, 336t
 classification of, 327t
- Streptococcus pneumoniae*, 36t, 333–334
 antigenic structure of, 333
 antimicrobial resistance and, 334
 bacterial meningitis and, 891–892
 classification of, 327t
 clinical infections of, 333
 colony morphology of, 179f
 differentiation of, 179f
 laboratory diagnosis of, 334, 334f
 penicillins for, 284–285, 285f
 susceptibility testing and, 284–285, 285f
 virulence factors of, 333
- Streptococcus pyogenes*, 36t, 38t, 180f, 327–330
 antigenic structure of, 327
 bacterial pharyngitis and, 328, 328b
 classification of, 327t
 clinical infections from, 328–329
 laboratory diagnosis of, 329–330
 necrotizing fasciitis and, 328–329
 poststreptococcal sequelae and, 329
 pyoderma infections and, 328
 serologic testing for, 208
 toxic shock syndrome and, 329
 virulence factors of, 327–328
- Streptococcus salivarius*
 characteristics of, 336t
 classification of, 327t
- Streptococcus* spp.
 biochemical identification of, 330, 331t, 340–344, 341f, 342f
 cell wall structure of, 325–326, 326f
 classification of, 327t
 clinical infections of, 337
 clinically significant, 326–340
 general characteristics of, 325–326, 325f
 group A, testing of throat samples for, 118
 hemolysis and, 326, 326b, 326t
 hemolytic patterns and, 340
 laboratory diagnosis of, 337–338
 Lancefield classification system and, 340
 noncultural identification of, 344–345
 immunoassays and, 344
 slide agglutination test and, 344, 344f
 susceptibility testing and, 345
 nucleic acid probe assays and, 344–345
 phenotype and biochemical characteristics of, 337–338, 337t
 physiologic characteristics of, 340
 virulence factors of, 337
- Streptococcus*-like organisms, 338–340
 Abiotrophia and *Granulicatella* spp. and, 338
 Aerococcus spp. and, 338
 clinically significant, 326–340
 Dolosicoccus and *Globicatella* spp. and, 338–339
 Dolosigranulum spp. and, 339
 Facklamia spp. and, 339
 Gemella spp. and, 339
 Lactococcus spp. and, 339
 Leuconostoc spp. and, 339, 339f
 Pediococcus spp. and, 339–340
 Vagococcus spp. and, 340
- Streptogramins, 253t–254t
- Streptolysin O (SLO), 327
- Streptolysin S, 328
- Streptomyces* spp., 362–363, 363t
- Streptomycin, 253t–254t, 575
- Strict anaerobes, 15
- Stridor, 803
- Stringency, 229
- Strobila, 687f
- Strongyloides* infection, 859
- Strongyloides stercoralis*, 693t, 694, 697–699, 698f, 699f, 700f
 cancer patients and, 978
- Subculturing, 171–172
- Subcutaneous layer, of skin, 833, 834f
- Subcutaneous mycoses, 602
 agents of, 607–610
- Subcutaneous phaeohyphomycosis, 608–609, 609b
- Suboptimal specimens, 130
- Substrata, 778
- Subterminal spores, 501, 502f
- Sudan ebolavirus (SUDV), 733, 765
- Sugars, 185
- Sulbactam, 263
- Sulfamethoxazole (SMZ), 258
 Streptococcus spp. and, 331t
- Sulfide-indole-motility (SIM) agar, 191
- Sulfonamides, 253t–254t
- Sulfur granules, 361, 362f
- Superficial candidiasis, 834–836
- Superficial mycoses, agents of, 603–604
- Superoxide anion, 498
- Superoxide dismutase, 498
- Suppurative lymph nodes, 394
- Suprapubic aspirate, specimen collection from, 125t–126t
- Suprapubic aspiration, 933, 937
- Sure-Vue Mono test kit, 209f
- Surface proteins, bacteria, 5f
- Surgical hand scrub, 75, 76t, 77
- Surgical site infections (SSIs), 52, 53t
- Surgical therapy, 529
- Surveillance
 cultures, 54
 infection control and, 53–55, 54b
 baseline data, 54
 data gathering, 54–55
 definition of, 53–54
 general or targeted, 54
- Susceptibility testing
 Aeromonas spp. and, 466–467, 467b
 of agents of bioterrorism, 286
 anaerobes and, 286–287, 527–529, 529t
 options, 528–529
 problems in, 528–529
 antifungal, 635–636
 antimicrobial, 271, 272b, 319–322
 automated, 293–295
 procedures in, 272–273
 QC for, 295–298, 296t, 297t, 298t
 reasons and indications for, 273–274
 selecting, 298–299
 special, 299–300, 299b–300b, 300t
 traditional, 275–282
 battery selection for, 274, 274b
 body site in, 273
 Bordetella and, 414–416
 Borrelia burgdorferi and, 538
 Borrelia recurrentis and, 537
 Campylobacter spp. and, 471
 dilution and, 276–279
 disk diffusion testing and, 279–282, 279f
 Enterococcus spp. and, 289–290, 289f
 Etest and, 286–287
 Haemophilus influenzae and, 285
 Haemophilus parainfluenzae and, 285
 Helicobacter pylori and, 286
 host status in, 273
 inoculum preparation and standardization for, 275–276
 Klebsiella pneumoniae carbapenemase, 291
 Legionella and, 411

Susceptibility testing (*Continued*)

- for leptospires, 536, 536b
- MBC test and, 299–300, 300t
- McFarland turbidity standards and, 275–276
- MRSA and, 287
- of *Mycobacterium tuberculosis*, 594–595
- Mycoplasma* spp. and, 567
- Neisseria gonorrhoeae*, *Neisseria meningitidis* and, 286
- oxacillin and, 277t, 287–288, 288b, 288f
- presence of other bacteria and quality of specimen in, 273
- rapid, 299
- reporting results of, 274–275
- SBT and, 300t, 302
- selection of agents for, 274–275, 275b
- synergy tests and, 300t, 302, 302b
- test performance of, 280–282
- time-kill assays and, 300t, 301–302
- Treponema pallidum* and, 541
- Ureaplasma* spp. and, 567
- UTIs, 937–940
- vancomycin resistance in *Staphylococcus aureus* and, 288–289
- Vibrio* spp. and, 463
- Svedberg, Theodor, 7
- Svedberg units, 7
- Swabs, 506, 506f, 549
 - from smears, 141, 141f
- Swarming, 175f
- Swellings, Calabar, 701–702
- Swine flu, 735
- SYBR Green, 234, 236–237
- Sycosis barbae, 840
- Sydenham chorea, 329
- Sylvatic cycle, 734
- Symbionts, 26
- Symbiosis, 26
- Synanamorphs, 600
- Syncephalastrum* spp., 617, 617f
- Synchronous lesion, 727
- Syncytia, 717–718
- Syncytium, 6
- Synergism, 302, 303f
- Synergy tests, 300t, 302, 302b
- Syphilis, 534, 848–849, 849f, 956b
 - clinical manifestations of, 957–958, 957f
 - congenital, 539, 958
 - endemic, 538, 541
 - epidemiology of, 956–957, 956f
 - laboratory diagnosis of, 958–959
 - latent, 958
 - overview of, 956–959
 - primary stage of, 539
 - secondary stage of, 539
 - serologic testing of, 219–220, 220f, 540
 - tertiary stage of, 539
 - treatment for, 959
- Systemic dermatophyte infections, 605
- Systemic inflammatory response syndrome, 905, 905t
- Systemic mycoses, 602
 - agents of, 610–615, 611t

T

- T2 magnetic resonance assay, 634
- Tache noire, 995
- typhus group of, 995–996
- Tachypnea, 910–911
- Tachyzoites, 675
- Taenia saginata*, 688
- Taenia solium*, 688
- Taenia* spp., 688–689
 - laboratory diagnosis of, 689, 690f
 - life cycles of, 688–689, 690f
- Tai Forest ebolavirus (TAFV), 765
- Talaromyces marneffeii*, 615
- Tamiflu, 736
- Tapeworms (cestodes), 687–691, 687f, 688t
- Taq* DNA polymerase, 234
- TaqMan probe, 234–237, 235f
- Target, 229
- Target site modification, 264–265
- Targeted surveillance, 54
- Tatumella*, 439
- Taxa, 6
- Taxonomy
 - classification in
 - by cellular type, 7
 - by phenotypic and genotypic characteristics, 7
 - nomenclature in, 6
- Tazobactam, 263
- Tease mount, 631
- Technical sensitivity, 116
- Technical specificity, 116
- Tedizolid, 253t–254t
- Teicoplanin, 253t–254t
- Telavancin, 253t–254t
- Teleomorph, 600
- Telithromycin, 259
- Tellurite reduction, 593, 593f
- Temperate phage, 23
- Temperature, 229
 - Mycobacterium* spp. and, 590
 - quality control and, 110
 - sterilization and disinfection and, 68
 - yeast identification and, 634
- Template, 229
- TEN. *See* Toxic epidermal necrolysis
- Terminal spores, 501, 502f
- Tester strains, 604–605
- Testing slow-growing, modified methods for, 284–287
- TestPack Strep A kit, 216f
- Tests
 - analytic analysis of, 116
 - clinical analysis of, 116
 - diagnostic laboratory, evaluation and interpretation of, 116
 - efficiency of, 118–119, 119b
 - interpretation of, 282–293
 - operational analysis of, 117–119
 - predictive values of, 117–118
 - validation of, 119–121
- Tetanospasmin, 502
- Tetanus, 502–503
- Tetracyclines, 253t–254t, 259
- Tetrahydrofolate (THF), 258
- Thayer-Martin agar, modified, 171t
- Thayer-Martin medium, 376t
- The Joint Commission (TJC), 105
- Therapeutic agents, nanotechnology to deliver, 268–269
- Thermal cycler, 232–233
- Thermal injuries, 97
- Thermometer calibration, 110–111
- Thermophiles, 7, 15
- Thermostable DNA polymerase, 232–233
- Thiamine requirement, 632
- Thioglycollate broth, 171t, 177–178, 178f, 179f, 508t
- Thiomargarita namibiensis*, 11
- Thiophene-2-carboxylic acid hydrazide, 593
- Thiosulfate citrate bile salt sucrose agar (TCBS agar), 461, 461f
- Threadworm, 697–698
- 3M Integrated Cycler, 234f
- Throat, specimen collection from, 125t–126t
- Throat swab, 549
- Thrush, 622–623
- Ticks, 993
 - arboviruses and, 728–729
 - RMSF and, 553
- Tier 1 agents, 759–760
- Tigecycline, 253t–254t, 259, 787
- Time-kill assays, 300t, 301–302, 302b
- Tinctures, 72
- Tinea capitis*, 836, 837f
- Tinea corporis*, 836, 837f
- Tinea cruris*, 836
- Tinea favosa*, 605
- Tinea imbricata*, 605
- Tinea nigra*, 604
- Tinea pedis*, 605, 836
- Tinea versicolor*, 603, 837, 838f
- Tinidazole, 952
- Tissue
 - amebae, 654–657, 655t
 - culture, 716
 - flagellates, 662–667, 663f
 - mycotic sample collection of, 628
 - parasites, examination of specimens for, 644–645
 - roundworm infections in, 700–706
 - specimens, 125t–126t, 506
 - stains, 630, 630b, 630f, 630t
- TJC. *See* The Joint Commission
- TLR. *See* Toll-like receptor
- Tobramycin, 253t–254t
- Togaviridae, 742–743
- Tolerance, 47, 260, 301
- Toll-like receptor (TLR), 43
- Toluidine red unheated serum test (TRUST), 958
- Topoisomerases, 258
- TORCH agents, 205
- Total quality management (TQM), 105
- Total surveillance programs, 54
- Toxic epidermal necrolysis (TEN), 313
- Toxic megacolon, 873
- Toxic shock syndrome (TSS), 310, 313, 863–864
 - streptococcal, 329
- Toxic shock syndrome toxin-1 (TSST-1), 310–311
- Toxin-mediated cutaneous disease, 863–864
- Toxocara canis*, 701
- Toxocara cati*, 701
- Toxoplasma gondii*, 36t, 674–677, 978
 - clinical infections of, 675
 - laboratory diagnosis of, 676–677, 677f
 - life cycle of, 675–676, 675f, 676f
- TQM. *See* Total quality management

- Tracheal cytotoxin, 412
 Tracheitis, bacterial, 393
 Trachoma, 545, 547f
 Trailing growth, 278–279
 Transcription, 21, 258
 Transcription-mediated amplification (TMA), 239
 Transcription-PCR (RT-PCR), 232–233
 Transduction, 22–23, 22f, 267
 Transfer RNA (tRNA), 21, 258–259
 Transformation, 22, 22f, 267
 Transfusion-transmitted virus (TTV), 743–744, 748–749
 Transgrow, 375
 Transient bacteremia, 906
 Transient biota, 76
 Transient microbiota, 27
 Transillumination, 173, 174f
 Translation, 21
 Translucent density, 175, 175f
 Transmissible spongiform encephalopathies (TSEs), 749
 Transmission, routes of, 32–35
 Transmission-based precautions, 79
 Transparent density, 175, 175f
 Transplant recipients, 722
 Transport media, 14–15, 128
 Transposons, 21–22, 251
 Traveler's diarrhea, 872, 881
 Escherichia coli causing, 422
 geographic distribution of, 883f
 TREK Diagnostic System, 196
 Trematodes, 682–687
Treponema pallidum, 205, 848–849, 957
Treponema pallidum subsp. *pallidum*, 538–541
 antimicrobial susceptibility for, 541
 clinical manifestation of, 538–539
 epidemiology of, 539
 laboratory diagnosis of, 540–541
 microscopic examination of, 540
 serologic test of, 540
 specimen collection and handling for, 540
 virulence factors of, 538
Treponema pallidum-particulate agglutination test (TP-PA test), 540
 Treponemal antibody tests, 958
 Treponemal tests, 540
 Treponemes, 538–541, 538f
 Tricarboxylic acid cycle, 18, 19f
Trichinella spiralis, 700, 700f, 701f
 Trichinosis, 1013
Trichoderma spp., 620, 620f
Trichomonas vaginalis, 659t, 661–662
 Trichomoniasis
 clinical manifestations of, 951–952, 951f
 epidemiology of, 951
 laboratory diagnosis of, 952, 952f
 overview of, 951–952
 treatment of, 952
 Trichophyton agars, 632
Trichophyton concentricum, 605
Trichophyton mentagrophytes, 606, 606f
Trichophyton rubrum, 606, 607f
Trichophyton schoenleinii, 605
Trichophyton tonsurans, 606–607
Trichosporon beigeli, 603–604
Trichosporon spp., 603–604, 604f
Trichuris trichiura, 693t, 694–695, 695f
 Triclosan, 74
 Trimethoprim (TMP), 253t–254t, 258
 Triple sugar iron agar (TSIA), 185–187, 186t, 187f, 441t, 475
 Trismus, 502
 Trisodium phosphate, 586
Tropheryma whippelii, 364
 Trophozoites, 640–641, 642f
 True pathogen, 31
Trueperella, 358
Trypanosoma brucei gambiense, 664
Trypanosoma brucei rhodesiense, 664
Trypanosoma cruzi, 664, 666–667
 clinical infections of, 666
 life cycles of, 666, 666f, 667f
Trypanosoma spp., 664–666
 clinical infections of, 664–665
 laboratory diagnosis of, 665–666, 666f
 life cycle of, 665, 665f
 Trypanosomiasis, 664, 997
 Trypomastigote, 645
 Trypticase soy broth, 713
 Tsetse fly, 664
 TSS. See Toxic shock syndrome
 TSST-1. See Toxic shock syndrome toxin-1
 T-strain mycoplasma, 565
Tsukamurella, 363t
 Tube coagulase test, 316, 318f
 Tube dilution test, 277
 Tuberculosis, 819–823, 821f
 blastomycosis and, 820
 chronic pneumonia and, 818b, 819–823, 820b
 extensively drug-resistant, 575
 extrapulmonary, 573
 histoplasmosis and, 820
 meningitis and, 573
 miliary, 573
 multidrug-resistant, 574–575
 NAATs and, 821
 primary, 572–573
 reactivation, 573
 treatment for, 574, 822–823
 Tuberculosis verrucosa cutis (TVC), 847
 Tuberculous meningitis, 895
 Tularemia, 34t, 406–407, 766, 766f, 828–829, 851
 cause of, 989
 clinical manifestations of, 990, 990b
 diagnosis of, 851
 epidemiology of, 989–990
 overview of, 989–990, 989b
 Tumbling motility, 356
 Turbidity, 177–178, 178f
 McFarland standards and, 275–276
 Turnaround time (TAT), 715–716
 Tween 80 hydrolysis, 592
 Type B gastritis, 468
 Typhoid fever, 434, 435f
 Typhus fever, 850
 route of transmission, 33f
 Typhus group, of *Rickettsia* spp., 553–554
 Tzanck cells, 715
 Tzanck smear, 715
- U**
 Ulcers, 835b
Ulocladium spp., 622, 622f
 Umbilicate elevation, 175, 175f
 Umbonate elevation, 175, 175f
 Umbrella motility, 357f
 Uncoating, 712
 Undulant fever, 990
 Undulating membrane, 661–662
 Uni-Gold Recombigen HIV test, 741
 Unsuspected bacteremia, 905
 Unweighted pair group of method with arithmetic averages (UPGMA), 244
 Upper respiratory tract infections, 790, 796–805, 797t, 828b
 Upper urinary tract infections (U-UTIs), 924, 925b, 931t
 Urban cycle, 734
 Urea breath test, 471, 879f
Ureaplasma parvum, 562
Ureaplasma spp., 558, 558b
 antimicrobial susceptibility for, 567
 clinical infections with, 560–562
 culture and, 563–566, 564t
 isolation and identification of, 564–566, 566b, 566f
 laboratory diagnosis of, 562–567
 media and, 564
 nucleic acid amplification tests for, 563
 result interpretation of, 567–568, 568t
 serologic diagnosis of, 566–567, 567t
 specimen collection and transport for, 562–563
Ureaplasma urealyticum, 559, 567t
 Urease test, 191–192, 192f, 194t, 632
 anaerobe identification and, 515
 Mycobacterium spp. and, 593, 593f
 yeast and, 634
 Urethra, specimen collection from, 125t–126t
 Urethral specimens, of parasites, 644
 Urethritis, 546–547, 782, 924, 925b, 945b
 causes of, 931t, 945
 clinical manifestations of, 946–947, 947f
 epidemiology of, 945, 946f
 gonococcal, 945
 laboratory diagnosis of, 947–948, 947f
 nongonococcal, 546–547, 562, 945
 overview of, 945–948
 treatment of, 948
 Urinary system, 923, 926t
 anatomy of, 923f
 Urinary tract infections (UTIs), 274, 922, 922b–923b, 923b, 924t, 925b, 926f, 939b
 age and, 925–927
 antibiograms, 938–940
 bacteremia and, 910
 bladder catheterization and, 927
 causative agents of, 929–931, 929b, 929t, 931t
 causes of, 928–931, 931t
 clinical signs and symptoms of, 927–928, 928f
 coagulase-negative staphylococci causing, 930t
 colony counts, historical background for, 931–932
 cultures for, 936, 938f, 939t
 epidemiology and risk factors for, 925–927, 926b
 Escherichia coli causing, 422
 fungi and, 930–931, 931b, 935
 gram-negative bacilli and, 929

- Urinary tract infections (UTIs) (*Continued*)
 gram-positive bacilli and, 930
 gram-positive cocci and, 929–930
 interpretation of results, 937
 laboratory diagnosis of, 931–934
 microbial detection of, 934–935
 pathogenesis of, 928–929
 pregnancy and, 927
Pseudomonas fluorescens, *Pseudomonas putida* and, 483
 renal transplantation and, 927
 sexually transmitted infections, 927
 specimen collection for, 932–934
Staphylococcus aureus and, 310
 susceptibility testing and, 937–940
- Urine
 additives to, 934
 comparative parameters for, 924*t*
Mycobacterium spp. and, 584–585
 screening methods for
 automated, 935, 936*t*
 manual, 934–935, 935*b*, 935*t*
 specimen collection from, 125*t*–126*t*, 126
 transport of, 932–933
 volume of, 933
- Urine antigen test, 411
- Urine specimens, of parasites, 644
- Urogenital diseases, *Chlamydia trachomatis* and, 546–547
- Urogenital parasites, specimens examined for, 643–644
- Urogenital specimens, mycotic sample collection of, 628
- Uropathogenic *Escherichia coli*, 422, 423*t*
- V**
- V factor, 391–392, 396, 396*f*
- Vaccines, 749–751
 for COVID-19, 750–751
 genital warts and, 968
 for influenza, 809
 for *Neisseria meningitidis* infection, 383
 for poliovirus, 750–751
 for smallpox, 750–751
- Vaccinia virus, 726–727, 768, 769*f*
- Vaginal specimens, of parasites, 644
- Vaginitis, 782
- Vaginosis, bacterial, 359
- Vagococcus* spp., 340
- Vancomycin, 253*t*–254*t*, 787
 gram-positive organisms and, 331*t*, 345
- Vancomycin agar screen plate, 288–289
- Vancomycin resistance, 288–289
 in *Enterococcus* spp., detection of, 290
- Vancomycin-intermediate *Staphylococcus aureus* (VISA), 288, 321–322
- Vancomycin-resistant enterococci (VRE), 288
- Vancomycin-resistant staphylococci, 321–322
- Vancomycin-resistant *Staphylococcus aureus* (VRSA), 288, 321–322
- VAP. *See* Ventilator-associated pneumonia
- Variable number of tandem repeats (VNTRs), 243
- Variant viruses, 735
- Varicella (chickenpox), 724
- Varicella-zoster virus (VZV), 724–725, 724*f*, 725*f*, 827, 854–855, 855*f*, 978
 lesions, 715
- Varicella-zoster virus infection, 724
- Varicellovirus*, 724
- Variola major, 768
- Variola minor, 768
- Variola virus, 727, 727*f*, 767–769, 768*f*
- Vasculitis, 849, 862–863, 863*f*
- Vectors, 662
- Veillonella* spp., 504–505
- Venereal Disease Research Laboratory (VDRL), 219–220, 540
 test, 958
- Venezuelan equine encephalitis (VEE), 743
- Ventilation, laboratory safety and, 583
- Ventilator-associated pneumonia (VAP), 52, 53*t*, 806*t*–807*t*, 815–817, 816*b*, 976–977
- Verification, antibiograms and, 298*t*
- Vero, 716–717
- Verotoxin, 424
- Verrucous dermatitis, 607
- VersaTREK Culture System, 588–589, 916
- Vesicles, 5*f*, 837
- Vi antigen, 419, 433
- Vibrio alginolyticus*, 460
- Vibrio cholerae*, 38*t*, 458–459, 459*f*
- Vibrio parahaemolyticus*, 459–460
- Vibrio* spp., 455*b*–456*b*, 456–463
 antigenic structure of, 457–458
 antimicrobial susceptibility for, 463
 clinical manifestations of, 456, 457*t*
 culture media of, 460–461
 definitive identification of, 461–463, 462*t*, 463*t*
 diarrhea and, 877–878, 886, 886*f*
 general characteristics of, 456–458
 isolation of, 135*t*
 key features of, 458*t*
 laboratory diagnosis of, 460–463
 microscopic morphology of, 456–457, 457*f*
 physiology of, 457, 457*f*
 presumptive identification of, 461, 461*b*
 serology of, 461–463
 skin infections from, 848
 specimen collection and transport of, 460
- Vibrio vulnificus*, 460, 460*b*
- Vietnamese time bomb, 763
- Vines, 177–178, 178*f*
- Viral antigenemia test, 722
- Viral hepatitis, 969–970, 969*f*, 970*f*
- Viral infections
 causes of, 711*t*–712*t*
 DFA test in, 715
 diagnostic virology, methods in, 714–718
 enzyme immunoassays in, 715, 715*f*
 laboratory diagnosis of, 713–718
 meningitis and, 893–895, 893*b*, 893*f*, 894*b*, 894*f*, 901
 nucleic acid-based detection in, 715–716
 skin and soft tissue infections and, 853–858
 specimen collection and transport of, 713
 treatment and management of, 749–751
- Viral pathogens, diarrhea and, 875, 875*b*
- Viral replication, penetration and, 712
- Viral serology, 713
- Viral shedding, 718–719
- Viral zoonotic diseases, 997
- Viremia, 917
- Viridans streptococci, 334–336
 characteristics of, 336*t*
 clinical infections of, 335
 laboratory diagnosis of, 335–336
 virulence factors of, 335
- Virion, 6–7, 710
- Virulence, 35
 ability to produce extracellular toxins and enzymes and, 37–39
 ability to survive intracellularly and proliferate and, 37
 adherence to host cells and tissues in, 35–37, 36*f*
 of *Bacillus anthracis*, 364–365
 of *Bordetella pertussis*, 412
 of *Corynebacterium diphtheriae*, 349
 of Enterobacterales, 419
 of *Haemophilus influenzae*, 392–393
 of *Legionella*, 407–408
 of *Listeria monocytogenes*, 355
 microbial factors contributing to, 31–39
 of *Neisseria* species, 373, 374*f*
 of *Nocardia*, 361
 resistance to phagocytosis and, 33*t*, 35, 36*t*
 respiratory tract infections and, 795–796, 796*b*
 of *Salmonella*, 433
Vibrio parahaemolyticus and, 460
- Virulence factors
 of anaerobes, 500*t*
 of *Borrelia* spp., 536
 of Enterobacterales, 419
 of *Enterococcus* spp., 337
 microbial, 35
 of pathogenic organisms of ocular infections, 1003–1004, 1004*b*
 of *Pseudomonas aeruginosa*, 481–482, 482*f*, 482*t*
 of spirochetes, 534, 536
 of *Staphylococcus aureus*, 310–312, 312*t*
 of *Streptococcus agalactiae*, 330
 of *Streptococcus pneumoniae*, 333–334
 of *Streptococcus pyogenes*, 327–328
 of *Treponema pallidum*, 538
 of viridans streptococci, 335
- Viruses
 blepharitis and, 1008–1009, 1009*b*
 characteristics of, 710–713
 conjunctivitis and, 1007–1008
 culture of, 717*t*
 cytopathic effect of, 715
 dsDNA, 718–727
 dsRNA, 728–729
 hepatitis, 743–749
 isolation of, 716–718
 keratitis and, 1010, 1010*f*, 1011*b*
 laboratory diagnosis of, 713–718
 replication of, 710–713
 respiratory, 223
 respiratory tract infections and, 827
 serologic assays for, 718
 serologic testing of, 220–221
 ssDNA, 727–728
 ssRNA, 729–743
 structure of, 710
 taxonomy of, 710, 711*t*–712*t*
 variant, 735

- VISA. *See* Vancomycin-intermediate
Staphylococcus aureus
- Vision statement, 105
- VITEK systems, 195*t*, 196–197, 295, 295*f*
- Voges-Proskauer (VP) test, 187–188, 188*f*, 342–343
- Voided midstream specimen collection, 933
- Voriconazole, 635
- VRSA. *See* Vancomycin-resistant
Staphylococcus aureus
- Vulvovaginal candidiasis, 952
- Vulvovaginitis. *See also* Bacterial vaginosis (BV); Candidiasis; Trichomoniasis
causes of, 949
overview of, 949–953
- W**
- Wagatsuma agar, 460
- WalkAway system, 196
- Washings, *Mycobacterium* spp. and, 584
- Water, transmission by, 32–34
- Water sterility specimens, 136
- Waterborne pathogens, 60, 60*t*
- Waterhouse-Friderichsen syndrome, 381, 381*f*
- Waterless handrubs, surgical, 76–77, 76*b*
- Watson, James, 19
- Wautersiella* spp., 490
- Wee beasties, 4
- Weeksella* spp., 490
- Wee-Tab urease test tablet, 515
- Weighted pair group method with arithmetic averages (WPGMA), 244
- Weil disease, 535
- Weil syndrome, 991–992
- Weil-Felix agglutination test, 555
- West African sleeping sickness, 664
- West Nile fever, 734
- West Nile virus (WNV), 709, 716, 734, 894
- Western blot test, 218, 219*f*, 966
- Western equine encephalitis (WEE) virus, 743
- Wet mount preparations, 642
- Whipple disease, 364
- Whipworm, 694–695
- Whooping cough, 411
- Wide zone, 326*t*
- Widespread outbreaks, outbreak investigations and, 57–58, 58*f*
- Window period, 740
- Winterbottom sign, 664–665
- Wood lamp, 626, 836
- Woolsorter's disease, 365, 760, 988
- Work practice controls, 79
- Workflow, in laboratory, phases of, 103–105, 104*f*, 105*b*
- World War I, 755
- Worm burden, 682
- Worms
filarial, 701–703
guinea, 703
- Wound exudates, mycotic sample collection of, 628
- Wounds
Aeromonas spp. and, 464
botulism and, 502
specimen collection from, 125*t*–126*t*
Staphylococcus aureus and, 313
Vibrio vulnificus and, 460
- Wright stain, of *Yersinia pestis*, 771*f*
- Wright-Giemsa stain, 142–143, 143*t*
- Wuchereria bancrofti*, 701, 702*t*, 703*f*
- X**
- X factor, 391–392, 396, 396*f*, 397*f*
- Xenopsylla cheopis*, 769
- Xylose-lysine-deoxycholate (XLD) agar, 418, 440, 441*f*
- Y**
- Yaws, 541
route of transmission, 33*f*
- Yeast infections, 952. *See also* Candidiasis
agents of, 622–624, 624*f*, 624*t*
- Yeasts, 4–6, 598
carbohydrate assimilation and, 632–633
molds *versus*, 598–599
scum produced by, 177–178, 178*f*, 179*f*
test for identification of, 632–634
urease test and, 634
- Yellow fever virus, 733
route of transmission, 33*f*
- Yersin, Alexandre, 769, 992
- Yersinia*, 437–439
- Yersinia enterocolitica*, 437–438, 438*t*, 878
- Yersinia pestis*, 36*t*, 38*t*, 437, 438*t*, 985
bioterror agents, 769–771
clinical manifestations of, 769–770, 770*f*
direct examination of, 770–771, 771*f*
specimen collection and preparation of, 770
tests for presumptive identification of, 771
- Yersinia pseudotuberculosis*, 438–439, 438*t*
- Z**
- Zaire ebolavirus (ZEBOV), 765
- Zanamivir (Relenza), 736
- Zephiran, 586
- Zeta potential, 209
- Ziehl-Neelsen stain, 586, 821
- Ziemann dots, 671
- Zika virus, 710, 733–734
- Zone diameter interpretive breakpoints, 280, 280*f*
- Zone of equivalence, 206
- Zone of inhibition, 280
- Zoonoses, 32, 34, 34*t*, 404, 534, 849–851. *See also* Anthrax; Plague; *Rickettsia* spp.; Severe acute respiratory syndrome; Tularemia
- Anaplasmataceae, 996–997
- arthropods and, 992–997
- brucellosis, 990
- Capnocytophaga canimorsus* infection, 987, 987*b*
- cat scratch disease, 850, 987–988, 988*b*
- emerging, 998–999, 998*b*
- erysipeloid, 986–987, 986*b*, 987*f*
- leptospirosis, 850, 991–992, 991*b*, 992*b*
- Lyme borreliosis, 993–994, 993*b*, 993*f*
- overview of, 984, 985*b*
- pasteurellosis, 986, 986*b*
- rat bite fever, 850–851
- Rocky Mountain spotted fever and, 849, 994, 994*f*
- transmission
by direct contact or inhalation, 988–992
by scratches and bites, 986–988
- Zostavax, 725
- Zoster (shingles), 724
- Zygospores, 600*f*